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(57) Abstract

The present invention discloses novel methods for identifying drugs that can held regulate adiposity and fat content of animals, particularly in mammals. The discovery that PTP–1D binds to the phosphorylated leptin receptor when the receptor contains a phosphorylated tyrosine–985, provides a novel means for identifying agents to aid in the regulation of body weight and adiposity. Thus the present invention exploits this prior unknown role of PTP–1D by providing means for potentially treating and curing abnormalities of the endogenous leptin pathway, as well as allowing for the elected modification of body mass.

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METHODS OF IDENTIFYING AGENTS THAT MODULATE LEPTIN ACTIVITY

GOVERNMENTAL SUPPORT

The research leading to the present invention was supported, at least in part, by a grant from National Institute of Health, Grant No. DK41096. Accordingly, the Government may have certain rights in the invention.

FIELD OF THE INVENTION

The present invention relates to methods for identifying agents that can modulate the effect of the signal transduction pathway initiated by the natural hormone, leptin. Such agents can be used to aid the modulation of mammalian body mass. In particular, a new member of this signal transduction pathway has been identified. This new member, PTP-1D, has previously been identified as being a protein tyrosine phosphatase. The present invention discloses that the modulation of the phosphorylation state of PTP-1D can have an important effect on the biological activity of leptin. Therefore, the present invention has important therapeutic implications in homeostasis of body weight and fat tissue mass.

BACKGROUND OF THE INVENTION

Obesity, defined as an excess of body fat relative to lean body mass, is associated with important psychological and medical morbidities, the latter including hypertension, elevated blood lipids, and Type II or non-insulin-dependent diabetes melitis (NIDDM). There are 6-10 million individuals with NIDDM in the U.S., including 18% of the population of 65 years of age [Harris *et al.*, *Int. J. Obes.*, **11**:275-283 (1987)]. Approximately 45% of males and 70% of females with NIDDM are obese, and their diabetes is substantially improved or eliminated by weight reduction [Harris, *Diabetes Care*, **14(3)**:639-648 (1991)]. Both obesity and NIDDM are strongly

heritable, and the predisposing genes within the relevant controlled regulatory pathways are just beginning to be identified.

The assimilation, storage, and utilization of nutrient energy constitute a complex homeostatic system central to survival of metazoa. Among land-dwelling mammals, storage in adipose tissue of large quantities of metabolic fuel as triglycerides is crucial for surviving periods of food deprivation. The need to maintain a fixed level of energy stores without continual alterations in the size and shape of the organism requires the achievement of a balance between energy intake and expenditure. The identification of the relevant factors in the complex homeostasis system and the identification of agents that can be administered to control these factors is therefore of great importance.

An individual's level of adiposity is, to a large extent, genetically determined. Examination of the concordance rates of body weight and adiposity amongst mono- and dizygous twins or adoptees and their biological parents have suggested that the heritability of obesity (0.4-0.8) exceeds that of many other traits commonly thought to have a substantial genetic component, such as schizophrenia, alcoholism, and atherosclerosis [Stunkard *et al.*, *N. Engl. J. Med.*, **322**:1483-1487 (1990)]. Familial similarities in rates of energy expenditure have also been reported [Bogardus *et al.*, *Diabetes*, **35**:1-5 (1986)]. Genetic analysis in geographically delimited populations has suggested that a relatively small number of genes may account for the 30-50% of variance in body composition [Moll *et al.*, *Am. J. Hum. Genet.*, **49**:1243-1255 (1991)]. However, none of the genes responsible for obesity in the general population have been genetically mapped to a definite chromosomal location.

The most intensively studied mouse obesity mutations are the *ob* (obese) and *db* (diabetes) genes. Mice homozygous for either mutation are hyperphagic and hypometabolic, leading to an obese phenotype that is notable at one month of age. The weight of these animals tends to stabilize at 60-70 g (compared with 30-35 g in control mice).

Each of the rodent obesity models is accompanied by alterations in carbohydrate metabolism resembling those in Type II diabetes in man. In some cases, the severity of the diabetes depends in part on the background mouse strain [Leiter, *Endocrinology*, **124**:912-922 (1989)]. For both *ob* and *db*, congenic C57BL/Ks mice develop a severe diabetes with ultimate β cell necrosis and islet atrophy, resulting in a relative insulinopenia. Conversely, congenic C57BL/6J *ob* and *db* mice develop a transient insulin-resistant diabetes that is eventually compensated by β cell hypertrophy resembling human Type II diabetes.

The phenotype of *ob* and *db* mice resembles human obesity in ways other than the development of diabetes - the mutant mice eat more and expend less energy than do lean controls (as do obese humans). This phenotype is also quite similar to that seen in animals with lesions of the ventromedial hypothalamus, which suggests that both mutations may interfere with the ability to properly integrate or respond to nutritional information within the central nervous system.

A major advance in understanding the molecular basis for obesity occurred with the cloning of the *ob* gene. The mouse obesity (*ob*) gene encodes an adipose tissue-derived signaling factor for body weight homeostasis [Zhang *et al., Nature,* **372:**425 (1994); U.S. Patent Application No. 08/292,345 filed August 1, 1994; U.S. Patent Application No. 08/483,211, filed June 7, 1995, each of which is hereby incorporated by reference in its entirety]. The recombinant OB protein (leptin) purified from *Escherichia coli* can correct the obesity related phenotypes in *ob/ob* mice when exogenously administered [Campfield *et al., Science,* **269:**546 (1995); Pellymounter *et al., Science,* **269:**540 (1995); Halaas *et al., Science,* **269:**543 (1995); Stephens *et al., Nature,* **377:**530 (1995)]. Weight-reducing effects of the recombinant leptin were also observed in normal mice and mice with diet-induced obesity. Another advance occurred with the determination that the *db* gene encoded the leptin receptor [U.S. Patent Application No. 08/586,594 filed January 16, 1996; U.S. Patent Application No. 08/783,734 filed January 16, 1997; and Tartaglia *et al., Cell,* **83:**1263-1271 (1995) each of which is hereby incorporated by reference in its entirety].

Current studies suggest that obese humans and rodents (other than *ob/ob* mice) are not defective in their ability to produce leptin mRNA or protein and generally produce higher levels than lean individuals [Maffei *et al.*, *Nature Med.*, **1**:1155 (1995); Considine *et al.*, *J. Clin. Invest.*, **95**:2986 (1995); Lonnqvist *et al.*, *Nature Med.*, **1**:950 (1995); Hamilton *et al.*, *Nature Med.*, **1**:953 (1995)]. These data suggest that resistance to normal or elongated levels of leptin may be important factors in human obesity.

Leptin is a 16kD hormone that is the afferent signal in a negative feedback loop regulating food intake and body weight. The leptin receptor is a member of the cytokine family. Leptin's anorexigenic effect is dependent on binding to the Ob-Rb isoform of this receptor which encodes a long intracytoplasmic domain that includes several motifs for protein-protein interaction. Ob-Rb is highly expressed in the hypothalamus suggesting that this brain region is an important site of leptin action. Signal transduction by this class of receptor generally depends on ligand induced phosphorylation of soluble tyrosine receptor kinases such as JAK1, 2, 3, and tyk2. These kinases in turn phosphorylate tyrosine residues on the receptor which serve as docking sites for SH2 proteins. Phosphorylation of SH2 proteins after receptor binding initiates signal transduction. Leptin binds to a homodimer of the Ob-Rb isoform of its receptor thus activating JAK2. While the Stat3 transcription is activated by leptin *in vivo*, the identity of other components of this signal transduction pathway have not yet been identified.

Therefore there is a need to identify the components of the endogenous leptin pathway, *i.e.* the participants in the transduction of the signal initiated by the binding of the leptin to the leptin receptor. Furthermore, there is a need to design assays that can be used to identify agents that can modulate the endogenous leptin pathway by modulating the identified component.

The citation of any reference herein should not be construed as an admission that such reference is available as "Prior Art" to the instant application.

SUMMARY OF THE INVENTION

The present invention provides a method of identifying a modulator of the binding of a phosphorylated leptin receptor with PTP-1D. One such embodiment comprises contacting the phosphorylated leptin receptor or phosphorylated fragment thereof with PTP-1D or fragment thereof in the presence and absence of an agent under conditions in which in the absence of the agent, the binding of the phosphorylated leptin receptor or phosphorylated fragment with PTP-1D or fragment thereof can be detected. The binding of the phosphorylated leptin receptor or phosphorylated fragment thereof with PTP-1D or fragment thereof is then detected. When an increase in binding is detected in the presence of the agent, the agent is identified as a modulator that enhances the binding of the phosphorylated leptin receptor or phosphorylated fragment thereof with PTP-1D or fragment thereof. Alternatively, when a decrease in binding is detected in the presence of the agent, the agent is identified as a modulator that inhibits the binding of the phosphorylated leptin receptor or phosphorylated fragment thereof with PTP-1D or fragment thereof. In embodiments such as this one, the phosphorylated leptin receptor or phosphorylated fragment thereof contains a phosphorylated tyrosine-985, *i.e.*, the leptin receptor or the fragment thereof contains a tyrosine which is analogous to tyrosine 985 of SEQ ID NO:2 which is phosphorylated and therefore is a binding partner of PTP-1D.

In one such embodiment the phosphorylated leptin receptor or phosphorylated fragment thereof is bound to a solid support. In another such embodiment PTP-1D or fragment thereof is labeled. In still another embodiment the phosphorylated leptin receptor or phosphorylated fragment thereof is bound to a solid support and PTP-1D or fragment thereof is labeled. The present invention also includes analogous embodiments in which the phosphorylated leptin receptor or phosphorylated fragment thereof is labeled; and/or the PTP-1D or fragment thereof is bound to a solid support. As is exemplified below the phosphorylated fragment can also be part of a fusion (or chimeric) protein. In a preferred embodiment of this type the fusion protein comprises a phosphorylated fragment of the leptin receptor fused to a

glutathione-S-transferase. In another preferred embodiment of this type the fusion protein comprises a phosphorylated fragment of the leptin receptor fused to green fluorescent protein.

Another aspect of the present invention includes methods of identifying a modulator for the phosphorylated leptin receptor-dependent PTP-1D phosphorylation. One such embodiment comprises contacting a phosphorylated leptin receptor or phosphorylated fragment thereof with PTP-1D and JAK2 kinase in the presence and absence of an agent under conditions in which in the absence of the agent the phosphorylated leptin receptor or phosphorylated fragment thereof can stimulate the phosphorylation of PTP-1D. In such embodiments the phosphorylated leptin receptor or phosphorylated fragment thereof contains a phosphorylated tyrosine-985. Next the amount of phosphorylation of PTP-1D is determined. When either an increase or decrease in the phosphorylation is determined in the presence of the agent, relative to in its absence, the agent is identified as a potential modulator of the leptin receptor-dependent phosphorylation of PTP-1D. This embodiment can include the subsequent step of contacting of the potential modulator with PTP-1D under the initial conditions of contacting (*i.e.*, the first step) except a phosphorylated leptin receptor or phosphorylated fragment thereof containing the phosphorylated tyrosine-985 is not included. When no significant change in phosphorylation is determined in the presence of the potential modulator relative to in its absence, the potential modulator is identified as a modulator of the leptin-dependent phosphorylation of PTP-1D. In a preferred embodiment of this type, the phosphorylated fragment is part of a fusion protein.

In one such embodiment the fusion protein comprises the phosphorylated fragment fused to a glutathione-S-transferase. In another embodiment the fusion protein comprises the phosphorylated fragment fused to green fluorescent protein.

Another aspect of the present invention includes methods of identifying a potential modulator and/or modulator of the leptin-dependent PTP-1D phosphorylation *in situ*.

One such embodiment comprises contacting a cell with leptin in the presence and absence of an agent under conditions in which in the absence of the agent leptin induces the phosphorylation of PTP-1D. In such embodiments the cell comprises PTP-1D, JAK2, and a leptin receptor which contains tyrosine-985. The amount of phosphorylation of PTP-1D is then determined. When an increase or decrease in phosphorylation of PTP-1D is determined in the presence of the agent relative to in its absence, the agent is identified as a potential modulator of the leptin-dependent phosphorylation of PTP-1D.

In one such embodiment the cell is transfected with a vector which encodes either PTP-1D, JAK2, and a leptin receptor which contains tyrosine-985. In another embodiment the vector encodes two or more of the proteins. Alternatively, nucleic acids encoding two or more the proteins are transfected into the cell in two or more vectors individually encoding a protein. In a preferred embodiment the cell is transfected with a vector encoding PTP-1D, a vector encoding JAK2, and a vector encoding a leptin receptor which contains tyrosine-985.

In a preferred embodiment the method further comprises contacting a second cell with leptin and the potential modulator under the conditions of those of the initial step except in this case leptin cannot induce the phosphorylation of PTP-1D. The second cell comprises PTP-1D, JAK2, and a leptin receptor that does not contain tyrosine-985. The amount of phosphorylation of PTP-1D is then determined. When no significant change in phosphorylation is determined in the presence of the potential modulator relative to in its absence the potential modulator is identified as modulator of the leptin-dependent phosphorylation of PTP-1D.

In one particular embodiment the leptin receptor that does not contain tyrosine-985 is the Ob-Ra form of the receptor. In another particular embodiment the leptin receptor that does not contain tyrosine-985 is the Ob-Rb containing a phenylalanine-985 as exemplified below.

In one such embodiment the cell is transfected with a vector which encodes either PTP-1D, JAK2, and a leptin receptor which does not contains tyrosine-985. In another embodiment the vector encodes two or more of the proteins. Alternatively, nucleic acids encoding two or more the proteins are transfected into the cell in two or more vectors individually encoding a protein. In a preferred embodiment the cell is transfected with a vector encoding PTP-1D, a vector encoding JAK2, and a vector encoding a leptin receptor which does not contain tyrosine-985.

In one particular embodiment the modulator enhances the leptin receptor-dependent phosphorylation of PTP-1D. In another particular embodiment the modulator inhibits the leptin receptor-dependent phosphorylation of PTP-1D.

The present invention further provides a method of identifying a drug useful in a weight loss diet regimen. One such embodiment comprises administering one or more doses, (preferably multiple doses) to a test animal of an agent found to be an inhibitory modulator of the leptin-dependent phosphorylation of PTP-1D and a identical number of doses of a placebo to a control animal over a prescribed time period. The change in weight of the test animal and the control animal during the prescribed time period is then determined. An inhibitory modulator that causes the test animal to lose weight relative to the control animal is selected as a drug that is useful in a weight loss diet regimen.

The present invention further provides a method of identifying a drug useful in a weight gain diet regimen. One such embodiment comprises administering one or more doses, (preferably multiple doses) to a test animal of an agent found to be an enhancing modulator of the leptin-dependent phosphorylation of PTP-1D and a identical number of doses of a placebo to a control animal over a prescribed time period. The change in weight of the test animal and the control animal during the prescribed time period is then determined. An enhancing modulator that causes the test animal to gain weight relative to the control animal is selected as a drug that is useful in a weight gain diet regimen.

In either embodiment, the prescribed time period for laboratory animals being the test animal is preferably one week to two years, more preferably one month to one year, and even more preferably two months to six months.

Still another variation of the present invention includes a method of identifying a modulator of the leptin-dependent PTP-1D dephosphorylation of the JAK2 kinase *in situ*. One such embodiment comprises contacting a cell with leptin in the presence and absence of an agent under conditions in which in the absence of the agent, leptin induces the PTP-1D dephosphorylation of JAK2. The cell comprises PTP-1D, JAK2, and a leptin receptor which contains tyrosine-985. Next, the amount of phosphorylation of JAK2 is determined. When a decrease or increase in phosphorylation of JAK2 is determined in the presence of the agent relative to in its absence, the agent is identified as a potential modulator of the leptin-dependent PTP-1D dephosphorylation of JAK2. Then the cell is contacted with the potential modulator under the conditions of the initial step except in the absence of leptin. When no significant change in phosphorylation is determined in the presence of the potential modulator relative to in the absence of the potential modulator, the potential modulator is identified as a modulator of the leptin-dependent PTP-1D dephosphorylation of JAK2.

Again in one such embodiment the cell is transfected with a vector which encodes either PTP-1D, JAK2, and a leptin receptor which contains tyrosine-985. In another embodiment the vector encodes two or more of the proteins. Alternatively, nucleic acids encoding two or more the proteins are transfected into the cell in two or more vectors individually encoding a protein. In a preferred embodiment the cell is transfected with a vector encoding PTP-1D, a vector encoding JAK2, and a vector encoding a leptin receptor which contains tyrosine-985.

The present invention further includes a method of identifying an inhibitor of the leptin-dependent PTP-1D phosphorylation *in situ*. One such embodiment comprises (a)contacting a cell with leptin in the presence and absence of an agent under

conditions in which in the absence of the agent, leptin induces the expression of a reporter gene operably under the control of a promoter containing a binding site for activated Stat3. (b) The level of expression of the reporter gene contained by a host cell is determined. In this embodiment, the binding of activated Stat3 protein to the binding site induces the expression of the reporter gene in the cell comprises PTP-1D, JAK2, Stat3 and a leptin receptor which contains tyrosine-985. When the presence of the agent results in an increase in the level of expression of the reporter gene relative to in its absence, the agent is identified as a candidate inhibitor of the leptin-dependent PTP-1D phosphorylation. (c) Next, a second cell is contacted with leptin in the presence and absence of the candidate inhibitor under conditions in which in the absence of the candidate inhibitor leptin induces the phosphorylation of PTP-1D and wherein the second cell comprises PTP-1D, JAK2, and a leptin receptor which contains tyrosine-985. (d) The amount of phosphorylation of PTP-1D of step (c) is then determined. When a decrease in phosphorylation of PTP-1D is determined in the presence of the candidate inhibitor relative to in the absence of the candidate inhibitor, the candidate inhibitor is identified as a potential inhibitor of the leptin-dependent phosphorylation of PTP-1D. (e) A third cell is then contacted with leptin and the inhibitor under the conditions of those of step (c) except in this case leptin cannot induce the phosphorylation of PTP-1D. The third cell comprises PTP-1D, JAK2, and a leptin receptor that does not contain tyrosine-985. (f) The amount of phosphorylation of PTP-1D of step (e) then determined. When no decrease in phosphorylation is determined in the presence of the potential inhibitor relative to in its absence, the potential inhibitor is identified as an inhibitor of leptin-dependent phosphorylation of PTP-1D. Again the various proteins used in this assay can be native to the cell or preferably added by transfection of the cell with vectors encoding these proteins as described above.

In a preferred embodiment of this method, the reporter gene encodes luciferase. In a related embodiment the reporter gene encodes green fluorescent protein.

One aspect of this method further includes identifying a drug useful in a weight loss diet regimen. One such embodiment comprises administering one or more doses, (preferably multiple doses) to a test animal of an agent found to be an inhibitory modulator of the leptin-dependent phosphorylation of PTP-1D and a identical number of doses of a placebo to a control animal over a prescribed time period. The change in weight of the test animal and the control animal during the prescribed time period is then determined. An inhibitory modulator that causes the test animal to lose weight relative to the control animal is selected as a drug that is useful in a weight loss diet regimen.

The present invention further provides a method of identifying a drug useful in a weight gain diet regimen. One such embodiment comprises administering one or more doses, (preferably multiple doses) to a test animal of an agent found to be an enhancing modulator of the leptin-dependent phosphorylation of PTP-1D and a identical number of doses of a placebo to a control animal over a prescribed time period. The change in weight of the test animal and the control animal during the prescribed time period is then determined. An enhancing modulator that causes the test animal to gain weight relative to the control animal is selected as a drug that is useful in a weight gain diet regimen.

In either embodiment, the prescribed time period for laboratory animals being the test animal is preferably one week to two years, more preferably one month to one year, and even more preferably two months to six months.

These and other aspects of the present invention will be better appreciated by reference to the following drawings and Detailed Description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Binding of a 64kD protein to phosphotyrosine-985 of the Ob-Rb cytoplasmic region. Figure 1A is a schematic representation of the full length leptin

receptor (Ob-Rb) and shows the three segments used to make the three Glutathione-S-Transferase (GST) fusion fragments. The three GST-fragments are ObRbTyrl, ObRbTyr2 and ObRbTyr3 which are named for the reversibly phosphorylatable tyrosines of this region: Tyrosine-985, Tyrosine-1077, and Tyrosine-1138 respectively.

Figure 1B shows the GST fusion fragments of the peptides spanning each of the three cytoplasmic tyrosines (Y 985, 1077, 1138) which were expressed in bacteria with or without co-expression of the elk tyrosine kinase. Co-expression of elk tyrosine kinase led to the specific phosphorylation of these three tyrosine residues. The GST-Ob-Rb fragment 1 peptide (containing Y985) was incubated with protein extracts from bovine or mouse hypothalamus. After precipitation with anti-GST antibodies, the bound proteins were eluted, resolved on SDS PAGE, and stained with Coomassie Blue. A 64kD protein was found in the precipitates of mouse and bovine hypothalamus. The 64kD protein was not precipitated by the anti-GST antibody when tyrosine 985 was not phosphorylated. The 64kD protein was sequenced and found to be identical to Phosphostyrosine Phosphotase ID.

Figure 2: Immunoblots of the protein precipitate of GST-ObRb fragments. Figure 2A depicts an immunoblot using anti-PTP-1D antibody. Figure 2B depicts an immunoblot using anti-Stat3 antibody. The phosphorylated and unphosphorylated GST-ObRb fusion fragments 1 and 3 were incubated with protein extracts of mouse hypothalamus. PTP-1D was detected only in the material precipitated by the phosphorylated from of GST-ObRbTyr1. Stat3 was detected only in the proteins precipitated by the phosphorylated form of GST-ObRbTyr3.

Figure 3: Binding of Leptin to Ob-Rb leads to the Inducible Phosphorylation of PTP-1D. 293 cells were transfected using combinations of Ob-R, JAK2 and PTP-1D as indicated. The state of PTP-1D phosphorylation was assayed with and without leptin treatment. PTP-1D was first assayed by immunoprecipitation using an anti PTP-1D antibody followed by immunobloting using an anti-phosphotyrosine antibody. In

separate studies the total cell lysate was immunobloted with the anti-phosphotyrosine antibody. Treatment of cells with leptin transfected with Ob-Rb, JAK2 and PTP-1D led to an increase PTP-1D phosphorylation. This was confirmed using either method of detection. Transfection of Ob-Ra, JAK2 and PTP-1D led to the constitutive phosphorylation of PTP-1D but the level of phosphorylation was unchanged by leptin treatment (a and b indicated Ob-Ra and Ob-Rb respectively).

Figure 4: PTP-1D Phosphorylation by Ob-Rb is Dependent on Tyr 985. 293 cells were transfected with wild type Ob-Rb or a Y—>F 985 (*i.e.*, tyrosine to the phenylalanine substitution) mutant leptin receptor together with JAK2 and PTP-1D. The level of PTP-1D phosphorylation was scored at various times after leptin treatment. Leptin increased the level of PTP-1D phosphorylation only in the cells that received the wild type leptin receptor. PTP-1D was not phosphorylated in cells transfected with the Y—>F 985 mutant.

Figure 5: Phosphorylated PTP-1D Dephosphorylates JAK2. The level of phosphorylation of JAK2 was assayed after leptin treatment in cells transfected with the wild type and Y—>F 985 mutant. After leptin treatment, the level of JAK2 phosphorylation was five-fold greater in cells that received the wild type leptin receptor. When PTP-1D was cotransfected, the level of JAK2 phosphorylation was ten-fold greater in the cells that were transfected with the Y—>F 985 mutant leptin receptor. These data indicated that binding of leptin to Ob-Rb phosphorylation of PTP-1D leads to the dephosphorylation of JAK2.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides novel assays for agents which can potentially serve as drugs in the modulation of mammalian body mass. The present invention is based in part on the discovery that Protein Tyrosine Phosphatase 1D, PTP-1D, described in United States Patent No. 5,589,375 hereby incorporated by reference in its entirety, is involved in the endogenous leptin pathway. More specifically, PTP-1D has been

found to bind to the phosphorylated leptin receptor, when the receptor contains a phosphorylated tyrosine-985.

The discovery of the role of PTP-1D has important implications for the treatment of nutritional disorders including, but not limited to, obesity, weight loss associated with cancer and AIDS and the treatment of diseases associated with obesity such as hypertension, heart disease, and Type II diabetes. In addition, there are potential agricultural uses for the agents identified by the methods described herein in cases where one might wish to modulate the body weight of domestic animals.

Therefore, if appearing herein, the following terms shall have the definitions set out below

As used herein, "physiological obesity" and "physiologically obese" refer to excessive adipose tissue that is due at least in part to abnormalities in the endogenous leptin pathway, including abnormalities in the effective signaling initiated by the binding of leptin to the leptin receptor. Abnormalities in the endogenous leptin pathway may be manifested in a number of ways including an abnormal food intake, an abnormal activity level, or an abnormal body temperature. In addition, the present invention allows drugs to be identified which can modulate body mass completely independently of any inherent abnormality in the endogenous leptin pathway per se by augmenting or diminishing the natural effect of leptin.

As used herein, "leptin" encompasses biologically active variants of naturally occurring leptin, as well as biologically active fragments of naturally occurring leptin and variants thereof, and combinations of the preceding. Leptin is the polypeptide product of the *ob* gene as described in the International Patent Publication No. WO 96/05309, and the US Patent Application No. 08/483,211 to which it claims priority, each of which is incorporated herein by reference in its entirety. Putative analogs and fragments of leptin are reported in US Patent 5,521,283, US Patent 5,532,336 and

International Patent Publication No. PCT 96/22308 for International Application No. PCT/US96/ 01471, each of which is incorporated herein by reference in its entirety.

As used herein the terms "bound" or "binds" or "associates" or "associated" are meant to include all such specific interactions that result in two or more molecules showing a preference for one another relative to some third molecule. This includes processes such as covalent, ionic, hydrophobic and hydrogen bonding but does not include non-specific associations such solvent preferences.

As used herein, the phrase "conditions related to abnormalities of the endogenous leptin pathway" encompasses conditions and diseases due, at least in part, to abnormalities involving leptin as detailed above.

The term "medically assisting" is used herein as a manner of attending to the health care needs of a subject who has a particular problem (*e.g.*, an abnormality in the endogenous leptin pathway) which encompasses either diagnosing or treating that problem, and all combinations thereof. In one embodiment, the invention provides for medically assisting a mammalian subject suffering from an abnormality in the endogenous leptin pathway resulting in decreased leptin activity. In another embodiment, a mammalian subject may be suffering from an abnormality resulting in increased leptin activity. In each case, the decreased or increased leptin activity may be manifested as a pathological state, such as obesity (decreased leptin activity) or anorexia (increased leptin activity).

The term "detectable" is used broadly herein to include a factor that directly emits the detectable signal (such as a fluorescent molecule) an entity bound directly to that factor (such as an antibody carrying a fluorescent molecule) as well as a detectable target of the factor or the entity (such as an epitope of an antigen that reacts with the antibody carrying a fluorescent molecule). Thus a target may be termed detectable due to either its potential or realized association with either a factor that functions as a detectable label or a factor that is bound to a detectable label. Detectable labels,

include but not limited to an enzyme, a radioactive element, a biochemiluminescent, a chromophore that absorbs in the ultraviolet and/or visible and/or infrared region of the electromagnetic spectrum; and a fluorophore.

As defined herein, a "quantitative relationship" between two or more determinations of one or more substances, includes the relative absolute amounts, a relative percentage, a relative ratio, the difference, sum, multiple and/or quotient for two or more determinations; and can further include appropriate first or higher order equations that express the relationship between two or more determinations in a manner that can be understood by a person skilled in the art to which the present invention pertains.

A "determination" or "determining" as used herein is the result of an assay that includes an attempt to detect and/or the quantifying of the substance detected such as that expressed in a quantity or an amount of that substance.

As used herein the term "mono-specific antibody" describes an antibody to a protein raised against a particular peptide sequence in that protein and is meant to imply that the antibody is specific for an epitope that includes that particular sequence. A monospecific antibody may be either polyclonal or monoclonal.

As used herein a phosphorylated leptin receptor or phosphorylated fragment thereof contains a phosphorylated "tyrosine-985" or "Y985" when the receptor or fragment thereof contains the amino acid sequence of Amino Acid 983 to Amino Acid 990 of SEQ ID NO:2 and tyrosine-985 is phosphorylated. In a preferred embodiment, the receptor or fragment thereof contains the amino acid sequence of Amino Acid 971 to Amino Acid 1000 of SEQ ID NO:2 and tyrosine-985 is phosphorylated. In a more preferred embodiment, the receptor or fragment thereof contains the amino acid sequence of Amino Acid 961 to Amino Acid 1010 of SEQ ID NO:2 and tyrosine-985 is phosphorylated. As exemplified below, the fragment contained Amino Acid 965 to Amino Acid 1001 of SEQ ID NO:2 and tyrosine-985 is phosphorylated.

In analogous embodiments, a leptin receptor can contain a dephosphorylated tyrosine-985 (*i.e.*, a tyrosine that is not phosphorylated at position 985 of SEQ ID NO:2) or a phenylalanine at position 985 of SEQ ID NO:2 in place of the tyrosine. As used herein, the receptor containing such a substitution is referred to "F985" or the "F985 mutant receptor."

The OB receptor contains three important structural domains: an extracellular (or extracytoplasmic) domain, a transmembrane domain, and a cytoplasmic domain. The extracellular domain binds leptin. The transmembrane domain comprises a stretch of highly non-polar amino acid residues that localize to the hydrophobic region of the cell membrane. In this respect, the term transmembrane domain has its ordinary meaning in molecular and cellular biology. Finally, the cytoplasmic domain of an OB receptor of the invention may contain none, one, or two JAK-binding concensus sequences, termed "Box 1" and "Box 2". A receptor having "Box 1" and "Box 2" is believed competent for signal transduction via the JAK-Stat pathway upon binding of leptin.

In a further aspect, the OB-R polypeptide from one species is closely related (homologous) to the OB-R in another species. In particular, the human OB-R polypeptide is highly homologous to murine OB-R polypeptide. This observation is consistent with the data showing that human leptin is active in mice: for the hormone to be active interspecies, one would expect a high degree of similarity or homology between the receptors from different species as well.

In its primary aspect, the present invention is directed to the identification of agents that function as modulators of mammalian body weight.

The term "substantially similar" as used herein means at least 50% sequence similarity, preferably at least 60% sequence similarity, more preferably at least 70% sequence similarity, even more preferably at least 80% sequence similarity, and most preferably at least 90% sequence similarity.

The term "gene" as used herein refers to a nucleic acid, such as DNA, which codes on expression for a protein. Unless stated otherwise, gene may include mRNA, cDNA, or genomic DNA.

A composition comprising "A" (where "A" is a single protein, DNA molecule, vector, recombinant host cell, etc.) is substantially free of "B" (where "B" comprises one or more contaminating proteins, DNA molecules, vectors, etc., but excluding racemic forms of A) when at least about 75% by weight of the proteins, DNA, vectors (depending on the category of species to which A and B belong) in the composition is "A". Preferably, "A" comprises at least about 90% by weight of the A+B species in the composition, most preferably at least about 99% by weight. It is also preferred that a composition, which is substantially free of contamination, contain only a single molecular weight species having the activity or characteristic of the species of interest.

Contemplated by the present invention are analogs comprising conservative amino acid substitutions. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity, which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. In some instances, one polar amino acid may be substituted with another to preserve local hydrophilicity; more likely, a substitution that conserves charge, or at least does not introduce the opposite charge, is required. Such alterations will not be expected to affect apparent molecular weight as determined by polyacrylamide gel electrophoresis, or isoelectric point.

Particularly preferred substitutions are:

- Lys for Arg and vice versa such that a positive charge may be maintained;
- Glu for Asp and vice versa such that a negative charge may be maintained;
- Ser for Thr such that a free -OH can be maintained; and
- Gln for Asn such that a free NH_2 can be maintained.

Amino acid substitutions may also be introduced to substitute an amino acid with a particularly preferable property. For example, a Cys may be introduced a potential site for disulfide bridges with another Cys. A His may be introduced as a particularly "catalytic" site (i.e., His can act as an acid or base and is the most common amino acid in biochemical catalysis). Pro may be introduced because of its particularly planar structure, which induces β-turns in the protein's structure.

As used herein, the term "homologous" in all its grammatical forms refers to the relationship between proteins that possess a "common evolutionary origin," including proteins from superfamilies (*e.g.*, the immunoglobulin superfamily) and homologous proteins from different species (*e.g.*, myosin light chain, etc.) (Reeck et al., 1987, Cell 50:667). Such proteins have sequence homology as reflected by their high degree of sequence similarity.

Accordingly, the term "sequence similarity" in all its grammatical forms refers to the degree of identity or correspondence between nucleic acid or amino acid sequences of proteins that may or may not share a common evolutionary origin (*see* Reeck et al., *supra*). However, in common usage and in the instant application, the term "homologous," when modified with an adverb such as "highly," may refer to sequence similarity and not a common evolutionary origin.

The term "corresponding to" is used herein to refer similar or homologous sequences, whether the exact position is identical or different from the molecule to which the similarity or homology is measured. Thus, the term "corresponding to" refers to the

sequence similarity, and not the numbering of the amino acid residues or nucleotide bases.

A gene encoding the leptin receptor, leptin, PTP-1D or JAK2, whether genomic DNA or cDNA, can be isolated from any animal source, particularly from a mammal. Methods for obtaining the *Stat protein* gene are well known in the art, as described above (*see, e.g.*, Sambrook *et al.*, 1989, *supra*).

A "heterologous nucleotide sequence" as used herein is a nucleotide sequence that is added to a nucleotide sequence of the present invention by recombinant methods to form a nucleic acid which is not naturally formed in nature. Such nucleic acids can encode chimeric and/or fusion proteins. Thus the heterologous nucleotide sequence can encode peptides and/or proteins which contain regulatory and/or structural properties. In another such embodiment the heterologous nucleotide can encode a protein or peptide that functions as a means of detecting the protein or peptide encoded by the nucleotide sequence of the present invention after the recombinant nucleic acid is expressed. In still another such embodiment the heterologous nucleotide can function as a means of detecting a nucleotide sequence of the present invention. A heterologous nucleotide sequence can comprise non-coding sequences including restriction sites, regulatory sites, promoters and the like.

Nucleic Acids

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. *See, e.g.*, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989); Glover ed., *DNA Cloning: A Practical Approach*, Volumes I and II, MRL Press, Ltd., Oxford, U.K. (1985); Gait ed., Oligonucleotide Synthesis, Oxford University Press (1984); Hames *et al.*, eds., *Nucleic Acid Hybridization*, Springer-Verlag (1985); Hames *et al.*, eds. *Transcription And Translation*, Oxford University Press (1984); Freshney ed., *Animal Cell Culture*,

Oxford University Press (1986); *Immobilized Cells And Enzymes*, IRL Press (1986); Perbal, *A Practical Guide To Molecular Cloning*, Wiley, New York (1984). Of particular relevance to the present invention are strategies for isolating, cloning, sequencing, analyzing, and characterizing a gene or nucleic acid based on the well known polymerase chain reaction (PCR) techniques.

A "replicon" is any genetic element (*e.g.*, plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*, *i.e.*, capable of replication under its own control.

A "vector" is a replicon, such as a plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A "cassette" refers to a segment of DNA that can be inserted into a vector at specific restriction sites. The segment of DNA encodes a polypeptide of interest, and the cassette and restriction sites are designed to ensure insertion of the cassette in the proper reading frame for transcription and translation.

A cell has been "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell.

A "clone" is a population of cells derived from a single cell or common ancestor by mitosis.

A "nucleic acid molecule" refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules") in either single-stranded form, or a double-stranded helix. Double-stranded DNA-DNA, DNA-RNA and RNA-RNA helices are possible. The term nucleic acid molecule, and in particular DNA or RNA molecule, refers only to the primary and secondary structure of the molecule, and does not limit

it to any particular tertiary or quaternary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear or circular DNA molecules (*e.g.*, restriction fragments), plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5′ to 3′ direction along the nontranscribed strand of DNA (*i.e.*, the strand having a sequence homologous to the mRNA). A "recombinant DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation.

A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single-stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength (*see* Sambrook *et al.*, 1989, *supra*). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. For preliminary screening for homologous nucleic acids, low stringency hybridization conditions, corresponding to a T_m of 55°C, can be used, *e.g.*, 5x SSC, 0.1% SDS, 0.25% milk, and no formamide; or 30% formamide, 5x SSC, 0.5% SDS). Moderate stringency hybridization conditions correspond to a higher T_m, *e.g.*, 40% formamide, with 5x or 6x SCC. High stringency hybridization conditions correspond to the highest T_m, *e.g.*, 50% formamide, 5x or 6x SCC. Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater the value of T_m for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher T_m) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating T_m have been derived (*see* Sambrook *et al.*, 1989, *supra*, 9.50-0.51). For hybridization with shorter nucleic acids, *i.e.*, oligonucleotides, the position of mismatches becomes

more important, and the length of the oligonucleotide determines its specificity (*see* Sambrook *et al.*, 1989, *supra*, 11.7-11.8). Preferably a minimum length for a hybridizable nucleic acid is at least about 18 nucleotides; more preferably at least about 27 nucleotides; most preferably the length is at least about 36 nucleotides.

In a specific embodiment, the term "standard hybridization conditions" refers to a Tm of 55°C, using conditions as set forth above. In a preferred embodiment, the Tm is 60°C, in a more preferred embodiment, the Tm is 60°C.

"Homologous recombination" refers to the insertion of a foreign DNA sequence of a vector in a chromosome. Preferably, the vector targets a specific chromosomal site for homologous recombination. For specific homologous recombination, the vector will contain sufficiently long regions of homology to sequences of the chromosome to allow complementary binding and incorporation of the vector into the chromosome. Longer regions of homology, and greater degrees of sequence similarity, may increase the efficiency of homologous recombination.

A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide in a cell *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5′ (amino) terminus and a translation stop codon at the 3′ (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (*e.g.*, mammalian) DNA, and even synthetic DNA sequences. If the coding sequence is intended for expression in a eukaryotic cell, a polyadenylation signal and transcription termination sequence will usually be located 3′ to the coding sequence.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, terminators, and the like, that provide for the

expression of a coding sequence in a host cell. In eukaryotic cells, polyadenylation signals are control sequences.

A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then trans-RNA spliced and translated into the protein encoded by the coding sequence.

A DNA sequence is "operatively linked" to an expression control sequence when the expression control sequence controls and regulates the transcription and translation of that DNA sequence. The term "operatively linked" includes having an appropriate start signal (*e.g.*, ATG) in front of the DNA sequence to be expressed and maintaining the correct reading frame to permit expression of the DNA sequence under the control of the expression control sequence and production of the desired product encoded by the DNA sequence. If a gene that one desires to insert into a recombinant DNA molecule does not contain an appropriate start signal, such a start signal can be inserted upstream (5′) of and in reading frame with the gene.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3′ direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3′ terminus by the transcription initiation site and extends upstream (5′ direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

Antibodies

According to the invention, leptin receptor, PTP-1D, or the various proteins used in the fusion fragments described herein can be produced recombinantly or by chemical

synthesis, and may be used as an immunogen to generate antibodies that recognize the proteins. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library.

A molecule is "antigenic" when it is capable of specifically interacting with an antigen recognition molecule of the immune system, such as an immunoglobulin (antibody) or T cell antigen receptor. An antigenic polypeptide contains at least about 5, and preferably at least about 10, amino acids. An antigenic portion of a molecule can be that portion that is immunodominant for antibody or T cell receptor recognition, or it can be a portion used to generate an antibody to the molecule by conjugating the antigenic portion to a carrier molecule for immunization. A molecule that is antigenic need not be itself immunogenic, *i.e.*, capable of eliciting an immune response without a carrier.

An "antibody" is any immunoglobulin, including antibodies and fragments thereof, that binds a specific epitope. The term encompasses polyclonal, monoclonal, and chimeric antibodies, the last mentioned described in further detail in U.S. Patent Nos. 4,816,397 and 4,816,567, as well as antigen binding portions of antibodies, including Fab, $F(ab')_2$ and F(v) (including single chain antibodies). Accordingly, the phrase "antibody molecule" in its various grammatical forms as used herein contemplates both an intact immunoglobulin molecule and an immunologically active portion of an immunoglobulin molecule containing the antibody combining site. An "antibody combining site" is that structural portion of an antibody molecule comprised of heavy and light chain variable and hypervariable regions that specifically binds antigen.

Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contains the paratope, including those portions known in the art as Fab, Fab', $F(ab')_2$ and F(v), which portions are preferred for use in the therapeutic methods described herein.

Fab and $F(ab')_2$ portions of antibody molecules are prepared by the proteolytic reaction of papain and pepsin, respectively, on substantially intact antibody molecules by methods that are well-known. See for example, U.S. Patent No. 4,342,566 to Theofilopolous *et al.* Fab' antibody molecule portions are also well-known and are produced from $F(ab')_2$ portions followed by reduction of the disulfide bonds linking the two heavy chain portions as with mercaptoethanol, and followed by alkylation of the resulting protein mercaptan with a reagent such as iodoacetamide. An antibody containing intact antibody molecules is preferred herein.

The phrase "monoclonal antibody" in its various grammatical forms refers to an antibody having only one species of antibody combining site capable of immunoreacting with a particular antigen. A monoclonal antibody thus typically displays a single binding affinity for any antigen with which it immunoreacts. A monoclonal antibody may therefore contain an antibody molecule having a plurality of antibody combining sites, each immunospecific for a different antigen; *e.g.*, a bispecific (chimeric) monoclonal antibody.

The term "adjuvant" refers to a compound or mixture that enhances the immune response to an antigen. An adjuvant can serve as a tissue depot that slowly releases the antigen and also as a lymphoid system activator that non-specifically enhances the immune response [Hood *et al.*, in *Immunology*, p. 384, Second Ed., Benjamin/Cummings, Menlo Park, California (1984)]. Often, a primary challenge with an antigen alone, in the absence of an adjuvant, will fail to elicit a humoral or cellular immune response. Adjuvants include, but are not limited to, complete Freund's adjuvant, incomplete Freund's adjuvant, saponin, mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil or hydrocarbon emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (*bacille Calmette-Guerin*) and *Corynebacterium parvum*. Preferably, the adjuvant is pharmaceutically acceptable.

Various procedures known in the art may be used for the production of polyclonal antibodies for use in the invention. For the production of antibody, various host animals can be immunized by injection with any protein used in the present invention, including but not limited to rabbits, mice, rats, sheep, goats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (*bacille Calmette-Guerin*) and *Corynebacterium parvum*.

For preparation of monoclonal antibodies directed toward the proteins used in the invention any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used. These include but are not limited to the hybridoma technique originally developed by Kohler *et al.*, *Nature*, **256**:495-497 (1975), as well as the trioma technique, the human B-cell hybridoma technique [Kozbor *et al.*, *Immunology Today*, **4**:72 (1983)], and the EBV-hybridoma technique to produce human monoclonal antibodies [Cole *et al.*, in *Monoclonal Antibodies and Cancer Therapy*, pp. 77-96, Alan R. Liss, Inc., (1985)]. Immortal, antibody-producing cell lines can be created by techniques other than fusion, such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. [*See, e.g.*, M. Schreier *et al.*, "Hybridoma Techniques" (1980); Hammerling *et al.*, "Monoclonal Antibodies And T-cell Hybridomas" (1981); Kennett *et al.*, "Monoclonal Antibodies" (1980); *see also* U.S. Patent Nos. 4,341,761; 4,399,121; 4,427,783; 4,444,887; 4,451,570; 4,466,917; 4,472,500; 4,491,632; and 4,493,890].

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, *e.g.*, radioimmunoassay, ELISA (enzyme-linked immunosorbent assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion

assays, *in situ* immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), Western blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc. In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention.

The foregoing antibodies can be used in methods known in the art, *e.g.*, for Western blotting, imaging.

A solid support for use in the present invention will be inert to the reaction conditions for binding. A solid support for use in the present invention may have reactive groups in order to attach a binding partner, such as an antibody to the leptin receptor. In another embodiment, the solid support may be a useful chromatographic support, such as the carbohydrate polymers SEPHAROSE, SEPHADEX, and agarose. As used herein, a solid support is not limited to a specific type of support. Rather, a large number of supports are available and are known to one of ordinary skill in the art. Solid supports include silica gels, resins, derivatized plastic films, glass beads, cotton, plastic beads, alumina gels, magnetic beads, membranes (including but not limited to nitrocellulose, cellulose, nylon, and glass wool filters), plastic and glass dishes or wells, etc. For example, solid supports used for peptide or oligonucleotide synthesis can be used, such as polystyrene resin (*e.g.*, PAM-resin obtained from Bachem Inc., Peninsula Laboratories, etc.), POLYHIPE® resin (obtained from Aminotech, Canada), polyamide resin (obtained from Peninsula Laboratories), polystyrene resin grafted with polyethylene glycol (TentaGel®, Rapp Polymere, Tubingen, Germany) or polydimethylacrylamide resin (obtained from Milligen/Biosearch, California). The solid support can be formulated as a chromatography support, *e.g.*, in a column; it can

be used in suspension followed by filtration, sedimentation, magnetic association, or centrifugation; or by washing, as in a membrane, well, plastic film, etc.

Phage libraries for Drug Screening.

Phage libraries have been constructed which when infected into host *E. coli* produce random peptide sequences of approximately 10 to 15 amino acids [Parmley and Smith, Gene **73**:305-318 (1988), Scott and Smith, Science **249**:386-249 (1990)]. Specifically, the phage library can be mixed in low dilutions with permissive *E. coli* in low melting point LB agar which is then poured on top of LB agar plates. After incubating the plates at 37°C for a period of time, small clear plaques in a lawn of *E. coli* will form which represents active phage growth and lysis of the *E. coli*. A representative of these phages can be absorbed to nylon filters by placing dry filters onto the agar plates. The filters can be marked for orientation, removed, and placed in washing solutions to block any remaining absorbent sites. The filters can then be placed in a solution containing, for example, PTP-1D and the leptin receptor containing a phosphorylated tyrosine-985. After a specified incubation period, the filters can be thoroughly washed and developed for autoradiography. Plagues containing the phage that interfere with the PTP-1D leptin receptor binding can then be identified. These phages can be further cloned and then retested. Once the phages have been purified, the binding sequence contained within the phage can be determined by standard DNA sequencing techniques. Once the DNA sequence is known, synthetic peptides can be generated which represents these sequences.

Labels:

Suitable labels include enzymes, fluorophores (*e.g.*, fluorescene isothiocyanate (FITC), phycoerythrin (PE), Texas red (TR), rhodamine, free or chelated lanthanide series salts, especially Eu^{3+}, to name a few fluorophores), chromophores, radioisotopes, chelating agents, dyes, colloidal gold, latex particles, ligands (*e.g.*, biotin), and chemiluminescent agents. When a control marker is employed, the same or different labels may be used for the receptor and control marker.

In the instance where a radioactive label, such as the isotopes ^{3}H, ^{14}C, ^{32}P, ^{35}S, ^{36}Cl, ^{51}Cr, ^{57}Co, ^{58}Co, ^{59}Fe, ^{90}Y, ^{125}I, ^{131}I, and ^{186}Re are used, known currently available counting procedures may be utilized. In the instance where the label is an enzyme, detection may be accomplished by any of the presently utilized colorimetric, spectrophotometric, fluorospectrophotometric, amperometric or gasometric techniques known in the art.

Direct labels are one example of labels which can be used according to the present invention. A direct label has been defined as an entity, which in its natural state, is readily visible, either to the naked eye, or with the aid of an optical filter and/or applied stimulation, e.g. U.V. light to promote fluorescence. Among examples of colored labels, which can be used according to the present invention, include metallic sol particles, for example, gold sol particles such as those described by Leuvering (U.S. Patent 4,313,734); dye sole particles such as described by Gribnau et al. (U.S. Patent 4,373,932) and May et al. (WO 88/08534); dyed latex such as described by May, *supra*, Snyder (EP-A 0 280 559 and 0 281 327); or dyes encapsulated in liposomes as described by Campbell et al. (U.S. Patent 4,703,017). Other direct labels include a radionucleotide, a fluorescent moiety or a luminescent moiety. In addition to these direct labeling devices, indirect labels comprising enzymes can also be used according to the present invention. Various types of enzyme linked immunoassays are well known in the art, for example, alkaline phosphatase and horseradish peroxidase, lysozyme, glucose-6-phosphate dehydrogenase, lactate dehydrogenase, urease, these and others have been discussed in detail by Eva Engvall in Enzyme Immunoassay ELISA and EMIT in *Methods in Enzymology*, **70**. 419-439, 1980 and in U.S. Patent 4,857,453.

Suitable enzymes include, but are not limited to, alkaline phosphatase and horseradish peroxidase.

Other labels for use in the invention include magnetic beads or magnetic resonance imaging labels.

In another embodiment, a phosphorylation site can be created on an antibody of the invention for labeling with ^{32}P, *e.g.*, as described in European Patent No. 0372707 (application No. 89311108.8) by Sidney Pestka, or U.S. Patent No. 5,459,240, issued October 17, 1995 to Foxwell et al.

As exemplified herein, proteins, including antibodies, can be labeled by metabolic labeling. Metabolic labeling occurs during *in vitro* incubation of the cells that express the protein in the presence of culture medium supplemented with a metabolic label, such as [^{35}S]-methionine or [^{32}P]-orthophosphate. In addition to metabolic (or biosynthetic) labeling with [^{35}S]-methionine, the invention further contemplates labeling with [^{14}C]-amino acids and [^{3}H]-amino acids (with the tritium substituted at non-labile positions).

Chimeric and/or fusion proteins containing luciferase or green fluorescent protein [U.S. Patent No. 5625,048 issued April 29, 1997 and WO 97/26333 published July 24, 1997 are hereby incorporated by reference] can also be used. Similarly these proteins can be encoded by reporter genes under the control of a promoter having an activated Stat3 binding site.

Vectors containing the reporter gene as well as nucleic acids encoding the leptin receptor, JAK2, and/or PTP-1D can be introduced into the desired host cells by methods known in the art, *e.g.*, transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, lipofection (lysosome fusion), use of a gene gun, or a DNA vector transporter (see, *e.g.*, Wu *et al., J. Biol. Chem.*, **267**:963-967 (1992); Wu and Wu, *J. Biol. Chem.*, **263**:14621-14624 (1988); Hartmut *et al.*, Canadian Patent Application No. 2,012,311, filed March 15, 1990).

Drug Assays

Potential drugs can be obtained from any source including the large chemical libraries compiled by large chemical companies such as Merck, Eli Lilly, Hoffman La Roche,

Glaxo Burroughs Welcome, etc. or alternatively can be obtained from phage libraries as detailed above.

The drug assays of the present invention may use any of a large number of protocols known to those having skill in the art. Such assays can measure protein-protein binding and/or stability, protein phosphorylation and/or dephosphorylation and protein activation. For example, protein phosphorylation can be measured by ^{32}P, with antibodies specific for phosphotyrosine as exemplified below or indirectly by the activation of expression of a reporter gene as described above. In this case, the reporter gene is under the control of a promoter having a promoter element which recognizes activated Stat3. Examples of such binding elements include the promoter of acute phase proteins [Zhang *et al., Biochem. Biophys. Res. Comm.,* **237**:90-94 (1997)]; haptoglobin promoter [Kim *et al., J. Biol. Chem.,* **272**:14571-14579 (1997)]; those disclosed by Lamb *et al.* [*Nucleic Acids Res.,* **23**:3283-3289 (1995)] and the SIE, present in the c-*fos* promoter [Ruff-Jamison *et al., J. Biol. Chem.,* **269**:21933-21935 (1994)].

Drug screening assays may be performed in cells that naturally encode the proteins involved in the signal transduction pathway initiated by leptin, preferably a cell is used that is transfected with a plasmid encoding the proteins of interest. For example transient transfections can be performed with 50% confluent U3A cells using the calcium phosphate method as instructed by the manufacturer (Stratagene). Alternatively 293 cells can be used. In certain embodiments the cells can also be modified to contain one or more reporter genes, a heterologous gene encoding a reporter such as luciferase, green fluorescent protein or derivative thereof, chloramphenicol acetyl transferase, β-galactosidase, etc. Such reporter genes can be operable linked to a promoter comprising a Stat3 binding site. Assays for detecting the reporter gene products are readily available in literature for example, luciferase assays can be performed according to the manufacturer's protocol (Promega), and β-galactosidase assays can be performed as described by Ausubel *et al.* [in *Current Protocols in Molecular Biology,* J. Wiley & Sons, Inc. (1994)].

In one example, the transfection reaction can comprise the transfection of a cell with plasmids modified to contain PTP-1D, the leptin receptor, and the JAK2 kinase, such as a pcDNA3 plasmid (Invitrogen). A reporter plasmid that contains a reporter gene under the control of a Stat3 responding promoter can also be included. Although preparation of such plasmids is now routine in the art, many appropriate plasmids are commercially available, *e.g.*, a plasmid with β-galactosidase is available from Stratagene.

Therapeutics

The drugs identified in the present invention have significant therapeutic potential. Preferably, a therapeutically effective amount of such an agent is administered in a pharmaceutically acceptable carrier, diluent, or excipient.

The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similarly untoward reaction, such as gastric upset, dizziness and the like, when administered to a human. In one embodiment, as used herein, the term "pharmaceutically acceptable" may mean approved by a regulatory agency of the federal or a state government or listed in the *U.S. Pharmacopeia* or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in Martin, *Remington's Pharmaceutical Sciences*, 18th Ed., Mack Publishing Co., Easton, PA (1990).

The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to reduce by at least about 15%, preferably by at least 50%, more preferably

by at least 90%, and most preferably prevent, a clinically significant deficit in the activity, function and response of the host. Alternatively, a therapeutically effective amount is sufficient to cause an improvement in a clinically significant condition in the host. Modulation of leptin activity can be useful for reducing body weight (by increasing its activity) or increasing body weight (by decreasing its activity).

Reduction of leptin activity (by developing antagonists, inhibitors, use of neutralizing antibodies, or antisense molecules) should result in weight gain as might be desirable for the treatment of the weight loss associated with cancer, AIDS or anorexia nervosa.

Pharmaceutical Compositions

In another aspect of the present invention, pharmaceutical compositions of the drugs identified are envisioned. Such pharmaceutical compositions may be for administration by injection, or for oral, pulmonary, nasal or other forms of administration. In general, comprehended by the invention are pharmaceutical compositions comprising effective amounts of protein or derivative products of the invention together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer content (*e.g.*, Tris-HCl, acetate, phosphate), pH and ionic strength; additives such as detergents and solubilizing agents (*e.g.*, Tween 80, Polysorbate 80), anti-oxidants (*e.g.*, ascorbic acid, sodium metabisulfite), preservatives (*e.g.*, Thimersol, benzyl alcohol) and bulking substances (*e.g.*, lactose, mannitol); incorporation of the material into particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc., or into liposomes. Hylauronic acid or other anionic polymers may also be used. Such compositions may influence the physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the present proteins and derivatives [*See, e.g.*, Martin, *Remington's Pharmaceutical Sciences*, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042) pages 1435-1712 which is herein incorporated by reference]. The compositions may be prepared in liquid form, or may be in dried powder, such as lyophilized form.

Oral Delivery

Contemplated for use herein are oral solid dosage forms, which are described generally in Martin, *Remington's Pharmaceutical Sciences*, 18th Ed. (1990 Mack Publishing Co. Easton PA 18042) at Chapter 89, which is herein incorporated by reference. Solid dosage forms include tablets, capsules, pills, troches or lozenges, cachets or pellets. Also, liposomal or proteinoid encapsulation may be used to formulate the present compositions (as, for example, proteinoid microspheres reported in U.S. Patent No. 4,925,673). Liposomal encapsulation may be used and the liposomes may be derivatized with various polymers (e.g., U.S. Patent No. 5,013,556). A description of possible solid dosage forms for the therapeutic is given by Marshall, in *Modern Pharmaceutics*, Chapter 10, Banker and Rhodes ed., (1979), herein incorporated by reference.

The present invention may be better understood by reference to the following non-limiting Example, which is provided as exemplary of the invention. The following example is presented in order to more fully illustrate the preferred embodiments of the invention. It should in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLE

LEPTIN RECEPTOR ACTIVATION OF PTP-1D MODULATES OB-R SIGNAL TRANSDUCTION

Summary:

Binding of leptin to its receptor, Ob-Rb, leads to JAK2 dependent tyrosine phosphorylation of the cytoplasmic domain of receptor and SH2 binding proteins including the STAT3 transcription factor. The phosphotyrosine phosphatase PTP-1D was isolated from bovine and mouse hypothalamus by affinity chromatography using fragments from the Ob-Rb carboxyterminus and shown to be another component of the leptin signal transduction pathway. In vitro binding studies indicated that PTP-1D binds to a phosphotyrosine at position 985 of the Ob-Rb isoform of the leptin

receptor. Co-transfection of Ob-Rb, JAK2 and PTP-1D indicated that the addition of leptin leads to the rapid phosphorylation of PTP-1D. After leptin treatment of cells transfected with wild type receptor the level of JAK2 phosphorylation was decreased in the presence of PTP-1D. The level of JAK2 phosphorylation was not decreased after leptin treatment of cells transfected with PTP-1D and a mutant Y —> F 985 leptin receptor. These data suggest that activation of PTP-1D by the leptin receptor results in the dephosphorylates JAK2, and may therefore act to inhibit leptin signal transduction.

Introduction

Leptin is a 16kD hormone that has potent weight reducing effects in vivo. Available data indicate that leptin is the afferent signal in a negative feedback loop regulating food intake and body weight. The leptin receptor is a member of the cytokine family. Leptins anorexigenic effect are dependent on binding to the Ob-Rb isoform of its receptor. The Ob-Rb form of this receptor encodes a long intracytoplasmic domain that includes several motifs for protein-protein interaction. The other forms of this receptor have short cytoplasmic regions and are not capable of initiating signal transduction by themselves. Ob-Rb is highly expressed in the hypothalamus suggesting that this region of the brain is an important site of leptin action. A mutation that specifically ablates Ob-Rb expression in mutant diabetic mice results in obesity and complete leptin resistance. Signal transduction by this class of receptor generally depends on ligand induced phosphorylation of soluble tyrosine receptor kinases such as JAK1, 2, 3, and tyk2. These kinases in turn phosphorylate tyrosine residues on the receptor which serve as docking sites for SH2 proteins. Phosphorylation of SH2 proteins after receptor binding initiates signal transduction.

Results

Leptin binds to a homodimer of the Ob-Rb isoform of its receptor and thereby activates JAK2. While the Stat3 transcription factor is activated by leptin in vivo, the identity of other components of this signal transduction pathway have not yet been identified To isolate other proteins involved in leptin signaling, Glutathione-S-

Transferase (GST) was fused to three polypeptide fragments spanning each of the three tyrosine residues encoded in the cytoplasmic region of Ob-Rb. The amino acids on the receptor included in each of these three constructs are as indicated (Figure 1A). The three GST Ob-Rb fusion fragments were introduced into bacteria also expressing of the elk tyrosine kinase. The elk kinase led to the specific tyrosine phosphorylation of the Ob-Rb fragments. Treatment of the fusion fragments with thrombin followed by immunobloting with an anti phosphotyrosine antibody confirmed that only the Ob-Rb peptide sequence was phosphorylated. Each of the three fusion fragments were incubated with protein extracts from bovine and mouse hypothalamus. The bound proteins were precipitated, eluted and fractionated by SDS PAGE. A single protein band of 64kD was observed after Coomassie Blue staining of protein precipitated after incubation with the fusion protein spanning the first tyrosine at position 985 (Figure 1B). Precipitation of the 64kD protein was dependent on the presence of a phosphotyrosine at position 985 as the fusion fragment without a phosphotyrosine was not active. The 64 kD protein was sequenced and the sequence was found to match PTP-1D. PTP-1D is a phosphotyrosine phosphatase. The relative efficiency of binding of PTP-1D to the GST Ob-Rb fragment 1 (GST-ObRbTyr1) was compared to that of Stat3 for the GST fusion protein spanning fragment 3 (GST-ObRbTyr3). Stat3 is known to be activated by Ob-Rb in vivo and a Stat3 binding motif, YXXL, is found in the region of tyrosine 1138. Ob-Rb fragments 1 and 3 were incubated with hypothalamic extracts and immunoblotted using anti-Stat3 and anti PTP-1D specific antisera. PTP-1D immunoactivity was found specifically in the precipitate using the GST Ob-Rb fusion fragment 1 (Figure 2A) while Stat3 immunoactivity was found only in the material precipitated after incubation with GST Ob-Rb fragment 3 (Figure 2B). The intensity of the PTP-1D signal was much greater than that of Stat3.

These data suggested that PTP-1D might play a role in leptin signal transduction. cDNA clones encoding Ob-Rb, JAK2, PTP-1D, and Ob-Ra were transfected in various combinations into 293 cells (Figure 3). Ob-Ra is a short form of the leptin receptor that does not encode any tyrosine residues in its 34 amino acid carboxyterminus. The transfected cells were treated with either PBS or leptin. In

each case the cellular proteins were immunoprecipitated using a specific anti-PTP-1D antibody and detected using an anti-phosphotyrosine antibody. In separate experiments a total cell lysate was immunoblotted using the anti-phosphotyrosine antibody. Phosphorylation of PTP-1D was observed only in the cells that were co-transfected with Ob-R and JAK2 (Figure 3). In the untreated Ob-Rb transfected cells, there was a low level of phosphorylation of PTP-1D that increased six fold when leptin was added to the culture. Curiously the basal level of phosphorylation was higher in the cells that were transfected with Ob-Ra but PTP-1D phosphorylation was not increased by the addition of leptin. Thus leptin binding to Ob-Rb leads to the phosphorylation of PTP-1D. The basis for the leptin independent activation of PTP-1D in the Ob-Ra transfected cells is unknown.

The time course of PTP-1D activation by leptin in Ob-Rb transfected cells was compared between wild type and a Y—>F 985 mutant receptor (Figure 4). Leptin induced PTP-1D phosphorylation in the cells transfected with the wild type receptor within 5 minutes and PTP-1D remained phosphorylated after one hour. The level of phosphorylation was substantially reduced in the cells transfected with the mutant receptor at all time points.

The possible function of PTP-1D in signal transduction was further assessed by following the time course and level of phosphorylation of the various components of the leptin signal transduction pathway. Cells were transfected with either the wild type or Y—>F 985 mutant receptor in the presence or absence of PTP-1D (Figure 5). Without PTP-1D, JAK2 was highly phosphorylated in response to leptin after 30 and 60 minutes of treatment. In this case, the level of phosphorylation of the wild type receptor was greater than that of the Y—>F 985 mutant receptor. When PTP-1D was added, this relationship was reversed and the level of JAK2 phosphorylation was much greater in the cells that were transfected with the mutant form of the receptor. These data suggest activation of PTP-1D after binding to Phosphotyrosine 985 leads to dephosphorylatase of JAK2. The present data indicate that PTP-1D is a component of the leptin signal transduction pathway. The data further indicate that when

activated, PTP-1D can dephosphorylate JAK2 and thereby inhibit the leptin signal transduction pathway. This role is analogous to that played by PTP-1C, another phosphotyrosine phosphotase, which dephosphorylates JAK2 in hematopoietic cells. Previous studies have suggested that PTP-1D acts as an activator of signal transduction. Thus corkscrew, the Drosophila homologue of PTP-1D, is required for signal transduction by the sevenless (DOS) receptor in R7 photoreceptor cells. In R7 cells corkscrew has been shown to lead to the dephosphorylation of daughter of sevenless leading to R7 differentiation. PTP-1D has also been suggested to play a positive role in signal transduction pathways activated by prolaction, IL3, PDGF and other peptide hormones. The data presented here indicate that in the setting of leptin signal transduction, PTP-1D acts as an inhibitor of signal transduction by decreasing the level of JAK2 phosphorylation. PTP-1D has also been suggested to reduce signal transduction in T cells that are inhibited by CTL-4.

Therefore, the inhibition of PTP-1D could enhance leptin action. However, PTP-1D function is not likely to be limited to leptin action as it is expressed ubiquitously. In addition, mice with induced mutations in PTP-1D are not viable and die after about 10.5 days of development. Finally, PTP-1D has also been shown to associate with the prolaction PDGF, 1L3, 1L4 and receptors. Nevertheless, PTP-1D appears to modulate leptin action in vivo.

The present invention is not be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

It is further understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

WHAT IS CLAIMED IS:

1. A method of identifying a modulator of the binding of a phosphorylated leptin receptor with PTP-1D comprising:

(a) contacting the phosphorylated leptin receptor or phosphorylated fragment thereof with PTP-1D or fragment thereof in the presence and absence of an agent under conditions in which in the absence of the agent the binding of the phosphorylated leptin receptor or phosphorylated fragment with PTP-1D or fragment thereof can be detected; and

(b) detecting the binding of the phosphorylated leptin receptor or phosphorylated fragment thereof with PTP-1D or fragment thereof; wherein when an increase in binding is detected in the presence of the agent, the agent is identified as a modulator that enhances the binding of the phosphorylated leptin receptor or phosphorylated fragment thereof with PTP-1D or fragment thereof, and when a decrease in binding is detected in the presence of the agent, the agent is identified as a modulator that inhibits the binding of the phosphorylated leptin receptor or phosphorylated fragment thereof with PTP-1D or fragment thereof; wherein the phosphorylated leptin receptor or phosphorylated fragment thereof contains a phosphorylated tyrosine-985.

2. The method of Claim 1 wherein the phosphorylated leptin receptor or phosphorylated fragment thereof is bound to a solid support.

3. The method of Claim 2 wherein PTP-1D or fragment thereof is labeled.

4. The method of Claim 1 wherein the phosphorylated fragment is part of a fusion protein.

5. The method of Claim 4 wherein the fusion protein comprises the phosphorylated fragment fused to a protein selected from the group consisting of glutathione-S-transferase, and green fluorescent protein.

6. A method of identifying a modulator of the phosphorylated leptin receptor-dependent PTP-1D phosphorylation comprising:

(a) contacting a phosphorylated leptin receptor or phosphorylated fragment thereof with PTP-1D and JAK2 kinase in the presence and absence of an agent under conditions in which in the absence of the agent the phosphorylated leptin receptor or phosphorylated fragment thereof stimulates the phosphorylation of PTP-1D; wherein the phosphorylated leptin receptor or phosphorylated fragment thereof contains a phosphorylated tyrosine-985;

(b) determining the amount of phosphorylation of PTP-1D; wherein when either an increase or decrease in the phosphorylation is determined in the presence of the agent relative to in the absence of the agent, the agent is identified as a potential modulator of the leptin receptor-dependent phosphorylation of PTP-1D; and

(c) contacting the potential modulator with PTP-1D under the conditions of step (a) except in the absence of the phosphorylated leptin receptor or phosphorylated fragment that contains a phosphorylated tyrosine-985; wherein when no significant change in phosphorylation is determined in the presence of the potential modulator relative to in the absence of the potential modulator, the potential modulator is identified as a modulator of the leptin-dependent phosphorylation of PTP-1D.

7. The method of Claim 6 wherein the phosphorylated fragment is part of a fusion protein.

8. The method of Claim 7 wherein the fusion protein comprises the phosphorylated fragment fused to a protein selected from the group consisting of glutathione-S-transferase, and green fluorescent protein.

9. A method of identifying a potential modulator and/or modulator of the leptin-dependent PTP-1D phosphorylation *in situ* comprising:

(a) contacting a cell with leptin in the presence and absence of an agent under conditions in which in the absence of the agent leptin induces the phosphorylation of PTP-1D; wherein the cell comprises PTP-1D, JAK2, and a leptin receptor which contains tyrosine-985; and

(b) determining the amount of phosphorylation of PTP-1D; wherein when an increase or decrease in phosphorylation of PTP-1D is determined in the presence of the agent relative to in the absence of the agent, the agent is identified as a potential modulator of the leptin-dependent phosphorylation of PTP-1D.

10. The method of Claim 9 wherein the cell is transfected with vectors which encode PTP-1D, JAK2, and a leptin receptor which contains tyrosine-985.

11. The method of Claim 9 further comprising:

(c) contacting a second cell with leptin and the potential modulator under the conditions of those of step (a) except wherein leptin cannot induce the phosphorylation of PTP-1D; and wherein the second cell comprises PTP-1D, JAK2, and a leptin receptor that does not contain tyrosine-985; and

(d) determining the amount of phosphorylation of PTP-1D; wherein when no significant change in phosphorylation is determined in the presence of the potential modulator relative to in the absence of the potential modulator, the potential modulator is identified as modulator of the leptin-dependent phosphorylation of PTP-1D.

12. The method of Claim 11 wherein the leptin receptor that does not contain tyrosine-985 is selected from the group consisting of Ob-Ra and Ob-Rb containing a phenylalanine-985.

13. The method of Claim 11 wherein the second cell is transfected with vectors which encode PTP-1D, JAK2, and the leptin receptor that does not contain tyrosine-985.

14. The method of Claim 11 wherein the modulator enhances the leptin receptor-dependent phosphorylation of PTP-1D.

15. The method of Claim 11 wherein the modulator inhibits the leptin receptor-dependent phosphorylation of PTP-1D.

16. A method of identifying a drug useful in a weight loss diet regimen comprising:

(a) administering multiple doses of the modulator of Claim 15 to a test animal and multiple doses of a placebo to a control animal over a prescribed time period; and

(b) determining the change in weight of the test animal and the control animal during the prescribed time period; wherein a modulator that causes the test animal to lose weight relative to the control animal is selected as a drug that is useful in a weight loss diet regimen.

17. A method of identifying a modulator of the leptin-dependent PTP-1D dephosphorylation of the JAK2 kinase *in situ* comprising:

(a) contacting a cell with leptin in the presence and absence of an agent under conditions in which in the absence of the agent, leptin induces the PTP-1D dephosphorylation of JAK2; wherein the cell comprises PTP-1D, JAK2, and a leptin receptor which contains tyrosine-985;

(b) determining the amount of phosphorylation of JAK2; wherein when a decrease or increase in phosphorylation of JAK2 is determined in the presence of the agent relative to in the absence of the agent, the agent is identified as a potential modulator of the leptin-dependent PTP-1D dephosphorylation of JAK2; and

(c) contacting the cell with the potential modulator under the conditions of step (a) but in the absence of leptin; wherein when no significant change in phosphorylation is determined in the presence of the potential modulator relative to in the absence of the potential modulator, the potential modulator is identified as a modulator of the leptin-dependent PTP-1D dephosphorylation of JAK2.

18. A method of identifying an inhibitor of the leptin-dependent PTP-1D phosphorylation *in situ* comprising:

(a) contacting a first cell with leptin in the presence and absence of an agent under conditions in which in the absence of the agent leptin induces the expression of a reporter gene operably under the control of a promoter containing a binding site for activated Stat3;

(b) measuring the level of expression of the reporter gene contained by the first cell; wherein the binding of activated Stat3 protein to the binding site induces the expression of the reporter gene; wherein the cell comprises PTP-1D, JAK2, Stat3 and a leptin receptor which contains tyrosine-985; and wherein when the presence of the agent results in an increase in the level of expression of the reporter gene relative to in the absence of the agent, the agent is identified as a candidate inhibitor of the leptin-dependent PTP-1D phosphorylation;

(c) contacting a second cell with leptin in the presence and absence of the candidate inhibitor under conditions in which in the absence of the candidate inhibitor leptin induces the phosphorylation of PTP-1D; wherein the second cell comprises PTP-1D, JAK2, and a leptin receptor which contains tyrosine-985;

(d) determining the amount of phosphorylation of PTP-1D of step (c); wherein when a decrease in phosphorylation of PTP-1D is determined in the presence of the candidate inhibitor relative to in the absence of the candidate inhibitor, the candidate inhibitor is identified as a potential inhibitor of the leptin-dependent phosphorylation of PTP-1D;

(e) contacting a third cell with leptin and the inhibitor under the conditions of those of step (c) except wherein leptin cannot induce the phosphorylation of PTP-1D; and wherein the third cell comprises PTP-1D, JAK2, and a leptin receptor that does not contain tyrosine-985; and

(f) determining the amount of phosphorylation of PTP-1D of step (e); wherein when no decrease in phosphorylation is determined in the presence of the potential inhibitor relative to in the absence of the potential inhibitor, the potential inhibitor is identified as an inhibitor of leptin-dependent phosphorylation of PTP-1D.

19. The method of Claim 18 wherein the reporter gene encodes a protein selected from the group consisting of luciferase and green fluorescent protein.

20. A method of identifying a drug useful in a weight loss diet regimen comprising:

(a) administering multiple doses of the inhibitor of Claim 18 to a test animal and multiple doses of a placebo to a control animal over a prescribed time period; and

(b) determining the change in weight of the test animal and the control animal during the prescribed time period; wherein an inhibitor that causes the test animal to lose weight relative to the control animal is selected as a drug that is useful in a weight loss diet regimen.

SEQUENCE LISTING

<110> Li, Cai

Friedman, Jeffrey M.

<120> METHODS OF IDENTIFYING AGENTS THAT MODULATE LEPTIN ACTIVITY

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385 390 395 400

Gly Lys Phe Thr Tyr Asp Ala Val Tyr Cys Cys Asn Glu Gln Ala Cys
405 410 415

His His Arg Tyr Ala Glu Leu Tyr Val Ile Asp Val Asn Ile Asn Ile
420 425 430

Ser Cys Glu Thr Asp Gly Tyr Leu Thr Lys Met Thr Cys Arg Trp Ser
435 440 445

Pro Ser Thr Ile Gln Ser Leu Val Gly Ser Thr Val Gln Leu Arg Tyr
450 455 460

His Arg Arg Ser Leu Tyr Cys Pro Asp Ser Pro Ser Ile His Pro Thr
465 470 475 480

Ser Glu Pro Lys Asn Cys Val Leu Gln Arg Asp Gly Phe Tyr Glu Cys
485 490 495

Val Phe Gln Pro Ile Phe Leu Leu Ser Gly Tyr Thr Met Trp Ile Arg
500 505 510

Ile Asn His Ser Leu Gly Ser Leu Asp Ser Pro Pro Thr Cys Val Leu

515 520 525

Pro Asp Ser Val Val Lys Pro Leu Pro Pro Ser Asn Val Lys Ala Glu
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Ile Thr Val Asn Thr Gly Leu Leu Lys Val Ser Trp Glu Lys Pro Val
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Phe Pro Glu Asn Asn Leu Gln Phe Gln Ile Arg Tyr Gly Leu Ser Gly
565 570 575

Lys Glu Ile Gln Trp Lys Thr His Glu Val Phe Asp Ala Lys Ser Lys
580 585 590

Ser Ala Ser Leu Leu Val Ser Asp Leu Cys Ala Val Tyr Val Val Gln
595 600 605

Val Arg Cys Arg Arg Leu Asp Gly Leu Gly Tyr Trp Ser Asn Trp Ser
610 615 620

Ser Pro Ala Tyr Thr Leu Val Met Asp Val Lys Val Pro Met Arg Gly
625 630 635 640

Pro Glu Phe Trp Arg Lys Met Asp Gly Asp Val Thr Lys Lys Glu Arg
645 650 655

Asn Val Thr Leu Leu Trp Lys Pro Leu Thr Lys Asn Asp Ser Leu Cys
660 665 670

Ser Val Arg Arg Tyr Val Val Lys His Arg Thr Ala His Asn Gly Thr
675 680 685

Trp Ser Glu Asp Val Gly Asn Arg Thr Asn Leu Thr Phe Leu Trp Thr
690 695 700

Glu Pro Ala His Thr Val Thr Val Leu Ala Val Asn Ser Leu Gly Ala
705 710 715 720

Ser Leu Val Asn Phe Asn Leu Thr Phe Ser Trp Pro Met Ser Lys Val
725 730 735

Ser Ala Val Glu Ser Leu Ser Ala Tyr Pro Leu Ser Ser Ser Cys Val
740 745 750

Ile Leu Ser Trp Thr Leu Ser Pro Asp Asp Tyr Ser Leu Leu Tyr Leu
755 760 765

Val Ile Glu Trp Lys Ile Leu Asn Glu Asp Asp Gly Met Lys Trp Leu
770 775 780

Arg Ile Pro Ser Asn Val Lys Lys Phe Tyr Ile His Asp Asn Phe Ile
785 790 795 800

Pro Ile Glu Lys Tyr Gln Phe Ser Leu Tyr Pro Val Phe Met Glu Gly
805 810 815

Val Gly Lys Pro Lys Ile Ile Asn Gly Phe Thr Lys Asp Ala Ile Asp
820 825 830

Lys Gln Gln Asn Asp Ala Gly Leu Tyr Val Ile Val Pro Ile Ile Ile
835 840 845

Ser Ser Cys Val Leu Leu Leu Gly Thr Leu Leu Ile Ser His Gln Arg
850 855 860

Met Lys Lys Leu Phe Trp Asp Asp Val Pro Asn Pro Lys Asn Cys Ser
865 870 875 880

Trp Ala Gln Gly Leu Asn Phe Gln Lys Pro Glu Thr Phe Glu His Leu
885 890 895

Phe Thr Lys His Ala Glu Ser Val Ile Phe Gly Pro Leu Leu Leu Glu
900 905 910

Pro Glu Pro Ile Ser Glu Glu Ile Ser Val Asp Thr Ala Trp Lys Asn
915 920 925

Lys Asp Glu Met Val Pro Ala Ala Met Val Ser Leu Leu Leu Thr Thr
930 935 940

Pro Asp Pro Glu Ser Ser Ser Ile Cys Ile Ser Asp Gln Cys Asn Ser
945 950 955 960

Ala Asn Phe Ser Gly Ser Gln Ser Thr Gln Val Thr Cys Glu Asp Glu
965 970 975

Cys Gln Arg Gln Pro Ser Val Lys Tyr Ala Thr Leu Val Ser Asn Asp
980 985 990

Lys Leu Val Glu Thr Asp Glu Glu Gln Gly Phe Ile His Ser Pro Val
995 1000 1005

Ser Asn Cys Ile Ser Ser Asn His Ser Pro Leu Arg Gln Ser Phe Ser

1010 1015 1020

Ser Ser Ser Trp Glu Thr Glu Ala Gln Thr Phe Phe Leu Leu Ser Asp
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Gln Gln Pro Thr Met Ile Ser Pro Gln Leu Ser Phe Ser Gly Leu Asp
1045 1050 1055

Glu Leu Leu Glu Leu Glu Gly Ser Phe Pro Glu Glu Asn His Arg Glu
1060 1065 1070

Lys Ser Val Cys Tyr Leu Gly Val Thr Ser Val Asn Arg Arg Glu Ser
1075 1080 1085

Gly Val Leu Leu Thr Gly Glu Ala Gly Ile Leu Cys Thr Phe Pro Ala
1090 1095 1100

Gln Cys Leu Phe Ser Asp Ile Arg Ile Leu Gln Glu Arg Cys Ser His
1105 1110 1115 1120

Phe Val Glu Asn Asn Leu Ser Leu Gly Thr Ser Gly Glu Asn Phe Val
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Pro Tyr Met Pro Gln Phe Gln Thr Cys Ser Thr His Ser His Lys Ile
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Met Glu Asn Lys Met Cys Asp Leu Thr Val
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<212> PRT

<213> Homo sapiens

<400> 4

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Phe Lys Leu Ser Cys Met Pro Pro Asn Ser Thr Tyr Asp Tyr Phe Leu
35 40 45

Leu Pro Ala Gly Leu Ser Lys Asn Thr Ser Asn Ser Asn Gly His Tyr
50 55 60

Glu Thr Ala Val Glu Pro Lys Phe Asn Ser Ser Gly Thr His Phe Ser
65 70 75 80

Asn Leu Ser Lys Thr Thr Phe His Cys Cys Phe Arg Ser Glu Gln Asp
85 90 95

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Arg Asn Cys Ser Leu Cys Ala Asp Asn Ile Glu Gly Lys Thr Phe Val
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Ser Thr Val Asn Ser Leu Val Phe Gln Gln Ile Asp Ala Asn Trp Asn
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Ile Gln Cys Trp Leu Lys Gly Asp Leu Lys Leu Phe Ile Cys Tyr Val
    130                 135                 140

Glu Ser Leu Phe Lys Asn Leu Phe Arg Asn Tyr Asn Tyr Lys Val His
145                 150                 155                 160

Leu Leu Tyr Val Leu Pro Glu Val Leu Glu Asp Ser Pro Leu Val Pro
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Gln Lys Gly Ser Phe Gln Met Val His Cys Asn Cys Ser Val His Glu
            180                 185                 190

Cys Cys Glu Cys Leu Val Pro Val Pro Thr Ala Lys Leu Asn Asp Thr
        195                 200                 205

Leu Leu Met Cys Leu Lys Ile Thr Ser Gly Gly Val Ile Phe Gln Ser
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Pro Leu Met Ser Val Gln Pro Ile Asn Met Val Lys Pro Asp Pro Pro
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Leu Gly Leu His Met Glu Ile Thr Asp Asp Gly Asn Leu Lys Ile Ser
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Trp Ser Ser Pro Pro Leu Val Pro Phe Pro Leu Gln Tyr Gln Val Lys
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Tyr Ser Glu Asn Ser Thr Thr Val Ile Arg Glu Ala Asp Lys Ile Val
275 280 285

Ser Ala Thr Ser Leu Leu Val Asp Ser Ile Leu Pro Gly Ser Ser Tyr
290 295 300

Glu Val Gln Val Arg Gly Lys Arg Leu Asp Gly Pro Gly Ile Trp Ser
305 310 315 320

Asp Trp Ser Thr Pro Arg Val Phe Thr Thr Gln Asp Val Ile Tyr Phe
325 330 335

Pro Pro Lys Ile Leu Thr Ser Val Gly Ser Asn Val Ser Phe His Cys
340 345 350

Ile Tyr Lys Lys Glu Asn Lys Ile Val Pro Ser Lys Glu Ile Val Trp
355 360 365

Trp Met Asn Leu Ala Glu Lys Ile Pro Gln Ser Gln Tyr Asp Val Val
370 375 380

Ser Asp His Val Ser Lys Val Thr Phe Phe Asn Leu Asn Glu Thr Lys
385 390 395 400

Pro Arg Gly Lys Phe Thr Tyr Asp Ala Val Tyr Cys Cys Asn Glu His
405 410 415

Glu Cys His His Arg Tyr Ala Glu Leu Tyr Val Ile Asp Val Asn Ile

420 425 430

Asn Ile Ser Cys Glu Thr Asp Gly Tyr Leu Thr Lys Met Thr Cys Arg
435 440 445

Trp Ser Thr Ser Thr Ile Gln Ser Leu Ala Glu Ser Thr Leu Gln Leu
450 455 460

Arg Tyr His Arg Ser Ser Leu Tyr Cys Ser Asp Ile Pro Ser Ile His
465 470 475 480

Pro Ile Ser Glu Pro Lys Asp Cys Tyr Leu Gln Ser Asp Gly Phe Tyr
485 490 495

Glu Cys Ile Phe Gln Pro Ile Phe Leu Leu Ser Gly Tyr Thr Met Trp
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Ile Arg Ile Asn His Ser Leu Gly Ser Leu Asp Ser Pro Pro Thr Cys
515 520 525

Val Leu Pro Asp Ser Val Val Lys Pro Leu Pro Pro Ser Ser Val Lys
530 535 540

Ala Glu Ile Thr Ile Asn Ile Gly Leu Leu Lys Ile Ser Trp Glu Lys
545 550 555 560

Pro Val Phe Pro Glu Asn Asn Leu Gln Phe Gln Ile Arg Tyr Gly Leu
565 570 575

Ser Gly Lys Glu Val Gln Trp Lys Met Tyr Glu Val Tyr Asp Ala Lys
580 585 590

Ser Lys Ser Val Ser Leu Pro Val Pro Asp Leu Cys Ala Val Tyr Ala
595 600 605

Val Gln Val Arg Cys Lys Arg Leu Asp Gly Leu Gly Tyr Trp Ser Asn
610 615 620

Trp Ser Asn Pro Ala Tyr Thr Val Val Met Asp Ile Lys Val Pro Met
625 630 635 640

Arg Gly Pro Glu Phe Trp Arg Ile Ile Asn Gly Asp Thr Met Lys Lys
645 650 655

Glu Lys Asn Val Thr Leu Leu Trp Lys Pro Leu Met Lys Asn Asp Ser
660 665 670

Leu Cys Ser Val Gln Arg Tyr Val Ile Asn His His Thr Ser Cys Asn
675 680 685

Gly Thr Trp Ser Glu Asp Val Gly Asn His Thr Lys Phe Thr Phe Leu
690 695 700

Trp Thr Glu Gln Ala His Thr Val Thr Val Leu Ala Ile Asn Ser Ile
705 710 715 720

Gly Ala Ser Val Ala Asn Phe Asn Leu Thr Phe Ser Trp Pro Met Ser
725 730 735

Lys Val Asn Ile Val Gln Ser Leu Ser Ala Tyr Pro Leu Asn Ser Ser
740 745 750

Cys Val Ile Val Ser Trp Ile Leu Ser Pro Ser Asp Tyr Lys Leu Met
755 760 765

Tyr Phe Ile Ile Glu Trp Lys Asn Leu Asn Glu Asp Gly Glu Ile Lys
770 775 780

Trp Leu Arg Ile Ser Ser Ser Val Lys Lys Tyr Tyr Ile His Asp His
785 790 795 800

Phe Ile Pro Ile Glu Lys Tyr Gln Phe Ser Leu Tyr Pro Ile Phe Met
805 810 815

Glu Gly Val Gly Lys Pro Lys Ile Ile Asn Ser Phe Thr Gln Asp Asp
820 825 830

Ile Glu Lys His Gln Ser Asp Ala Gly Leu Tyr Val Ile Val Pro Val
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Ile Ile Ser Ser Ser Ile Leu Leu Leu Gly Thr Leu Leu Ile Ser His
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Gln Arg Met Lys Lys Leu Phe Trp Glu Asp Val Pro Asn Pro Lys Asn
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Cys Ser Trp Ala Gln Gly Leu Asn Phe Gln Lys Pro Glu Thr Phe Glu
885 890 895

His Leu Phe Ile Lys His Thr Ala Ser Val Thr Cys Gly Pro Leu Leu
900 905 910

Leu Glu Pro Glu Thr Ile Ser Glu Asp Ile Ser Val Asp Thr Ser Trp

915 920 925

Lys Asn Lys Asp Glu Met Met Pro Thr Thr Val Val Ser Leu Leu Ser
930 935 940

Thr Thr Asp Leu Glu Lys Gly Ser Val Cys Ile Ser Asp Gln Phe Asn
945 950 955 960

Ser Val Asn Phe Ser Glu Ala Glu Gly Thr Glu Val Thr Tyr Glu Ala
965 970 975

Glu Ser Gln Arg Gln Pro Phe Val Lys Tyr Ala Thr Leu Ile Ser Asn
980 985 990

Ser Lys Pro Ser Glu Thr Gly Glu Glu Gln Gly Leu Ile Asn Ser Ser
995 1000 1005

Val Thr Lys Cys Phe Ser Ser Lys Asn Ser Pro Leu Lys Asp Ser Phe
1010 1015 1020

Ser Asn Ser Ser Trp Glu Ile Glu Ala Gln Ala Phe Phe Ile Leu Ser
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Asp Gln His Pro Asn Ile Ile Ser Pro His Leu Thr Phe Ser Glu Gly
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Leu Asp Glu Leu Leu Lys Leu Glu Gly Asn Phe Pro Glu Glu Asn Asn
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Asp Lys Lys Ser Ile Tyr Tyr Leu Gly Val Thr Ser Ile Lys Lys Arg
1075 1080 1085

Glu Ser Gly Val Leu Leu Thr Asp Lys Ser Arg Val Ser Cys Pro Phe
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Pro Ala Pro Cys Leu Phe Thr Asp Ile Arg Val Leu Gln Asp Ser Cys
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Ser His Phe Val Glu Asn Asn Ile Asn Leu Gly Thr Ser Ser Lys Lys
1125 1130 1135

Thr Phe Ala Ser Tyr Met Pro Gln Phe Gln Thr Cys Ser Thr Gln Thr
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<213> Mus musculus

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Pro Ser Lys Ser Asn Pro Gly Asp Phe Thr Leu Ser Val Arg Arg Asn
35 40 45

Gly Ala Val Thr His Ile Lys Ile Gln Asn Thr Gly Asp Tyr Tyr Asp
50 55 60

Leu Tyr Gly Gly Glu Lys Phe Ala Thr Leu Ala Glu Leu Val Gln Tyr
65 70 75 80

Tyr Met Glu His His Gly Gln Leu Lys Glu Lys Asn Gly Asp Val Ile
85 90 95

Glu Leu Lys Tyr Pro Leu Asn Cys Ala Asp Pro Thr Ser Glu Arg Trp
100 105 110

Phe His Gly His Leu Ser Gly Lys Glu Ala Glu Lys Leu Leu Thr Glu
115 120 125

Lys Gly Lys His Gly Ser Phe Leu Val Arg Glu Ser Gln Ser His Pro

130 135 140

Gly Asp Phe Val Leu Ser Val Arg Thr Gly Asp Asp Lys Gly Glu Ser
145 150 155 160

Asn Asp Gly Lys Ser Lys Val Thr His Val Met Ile Arg Cys Gln Glu
165 170 175

Leu Lys Tyr Asp Val Gly Gly Gly Glu Arg Phe Asp Ser Leu Thr Asp
180 185 190

Leu Val Glu His Tyr Lys Lys Asn Pro Met Val Glu Thr Leu Gly Thr
195 200 205

Val Leu Gln Leu Lys Gln Pro Leu Asn Thr Thr Arg Ile Asn Ala Ala
210 215 220

Glu Ile Glu Ser Arg Val Arg Glu Leu Ser Lys Leu Ala Glu Thr Thr
225 230 235 240

Asp Lys Val Lys Gln Gly Phe Trp Glu Glu Phe Glu Thr Leu Gln Gln
245 250 255

Gln Glu Cys Lys Leu Leu Tyr Ser Arg Lys Glu Gly Gln Arg Gln Glu
260 265 270

Asn Lys Asn Lys Asn Arg Tyr Lys Asn Ile Leu Pro Phe Asp His Thr
275 280 285

Arg Val Val Leu His Asp Gly Asp Pro Asn Glu Pro Val Ser Asp Tyr
290 295 300

Ile Asn Ala Asn Ile Ile Met Pro Glu Phe Glu Thr Lys Cys Asn Asn
305 310 315 320

Ser Lys Pro Lys Lys Ser Tyr Ile Ala Thr Gln Gly Cys Leu Gln Asn
325 330 335

Thr Val Asn Asp Phe Trp Arg Met Val Phe Gln Glu Asn Ser Arg Val
340 345 350

Ile Val Met Thr Thr Lys Glu Val Glu Arg Gly Lys Ser Lys Cys Val
355 360 365

Lys Tyr Trp Pro Asp Glu Tyr Ala Leu Lys Glu Tyr Gly Val Met Arg
370 375 380

Val Arg Asn Val Lys Glu Ser Ala Ala His Asp Tyr Thr Leu Arg Glu
385 390 395 400

Leu Lys Leu Ser Lys Val Gly Gln Ala Leu Leu Gln Gly Asn Thr Glu
405 410 415

Arg Thr Val Trp Gln Tyr His Phe Arg Thr Trp Pro Asp His Gly Val
420 425 430

Pro Ser Asp Pro Gly Gly Val Leu Asp Phe Leu Glu Glu Val His His
435 440 445

Lys Gln Glu Ser Ile Val Asp Ala Gly Pro Val Val Val His Cys Ser
450 455 460

Ala Gly Ile Gly Arg Thr Gly Thr Phe Ile Val Ile Asp Ile Leu Ile
465 470 475 480

Asp Ile Ile Arg Glu Lys Gly Val Asp Cys Asp Ile Asp Val Pro Lys
485 490 495

Thr Ile Gln Met Val Arg Ser Gln Arg Ser Gly Met Val Gln Thr Glu
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<213> Homo sapiens

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Gly Ala Val Thr His Ile Lys Ile Gln Asn Thr Gly Asp Tyr Tyr Asp
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Leu Tyr Gly Gly Glu Lys Phe Ala Thr Leu Ala Glu Leu Val Gln Tyr
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Asn Asp Gly Lys Ser Lys Val Thr His Val Met Ile Arg Cys Gln Glu
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Glu Ile Glu Ser Arg Val Arg Glu Leu Ser Lys Leu Ala Glu Thr Thr
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Asp Lys Val Lys Gln Gly Phe Trp Glu Glu Phe Glu Thr Leu Gln Gln
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Gln Glu Cys Lys Leu Leu Tyr Ser Arg Lys Glu Gly Gln Arg Gln Glu
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Asn Lys Asn Lys Asn Arg Tyr Lys Asn Ile Leu Pro Phe Asp His Thr
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Arg Val Val Leu His Asp Gly Asp Pro Asn Glu Pro Val Ser Asp Tyr
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Ile Asn Ala Asn Ile Ile Met Pro Glu Phe Glu Thr Lys Cys Asn Asn
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Thr Val Asn Asp Phe Trp Arg Met Val Phe Gln Glu Asn Ser Arg Val
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Val Arg Asn Val Lys Glu Ser Ala Ala His Asp Tyr Thr Leu Arg Glu
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Leu Lys Leu Ser Lys Val Gly Gln Gly Asn Thr Glu Arg Thr Val Trp
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Arg

Tyr Phosphorylated Ob-Rb Cytoplasmic Constructs

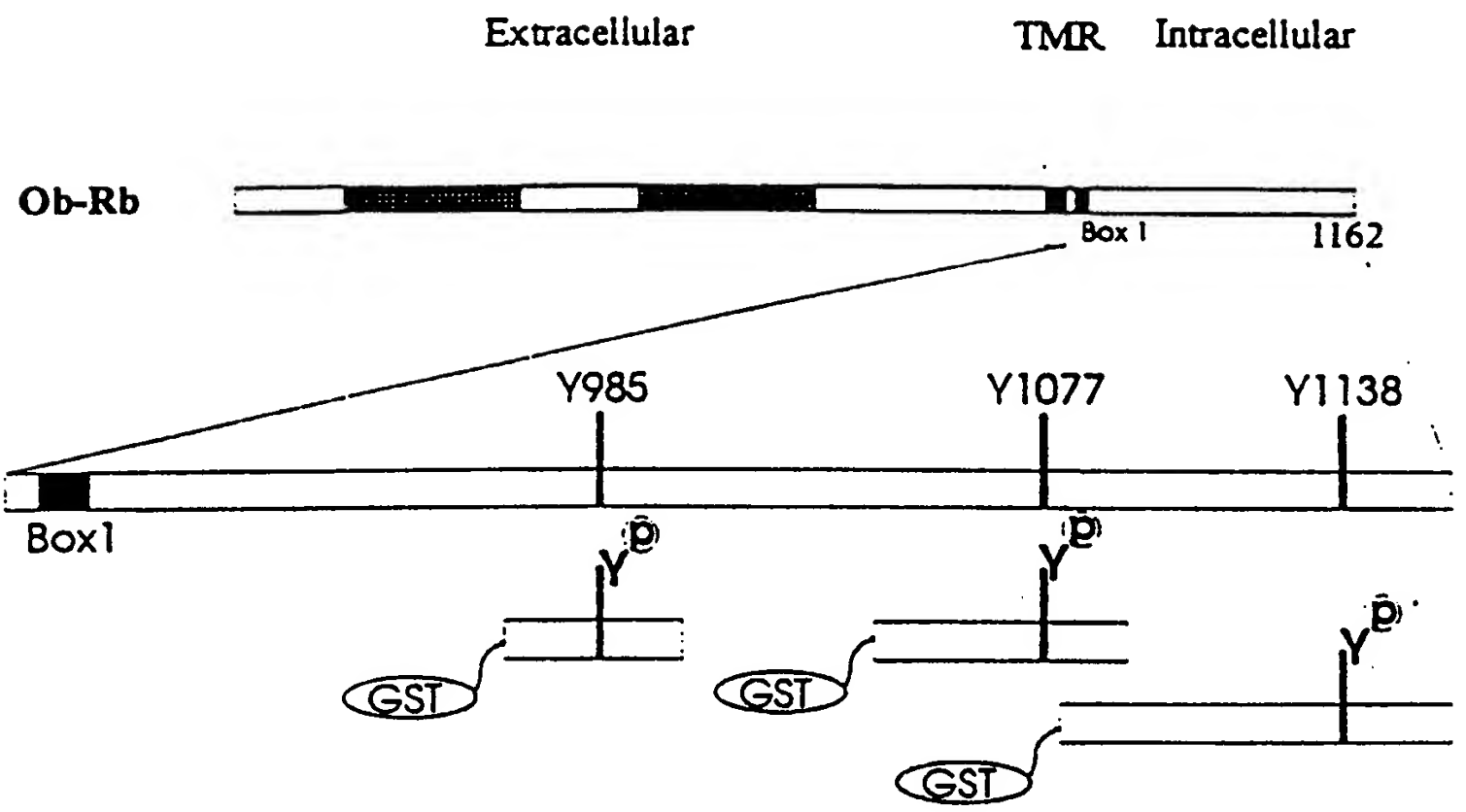
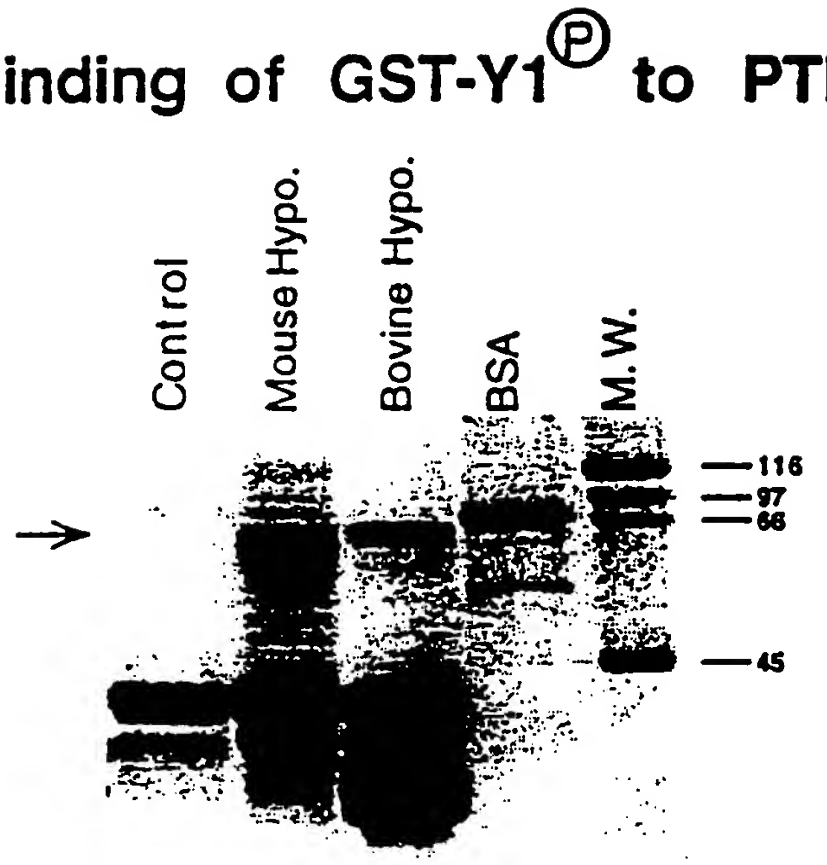


Figure 1A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Bovine Hypo.
BSA
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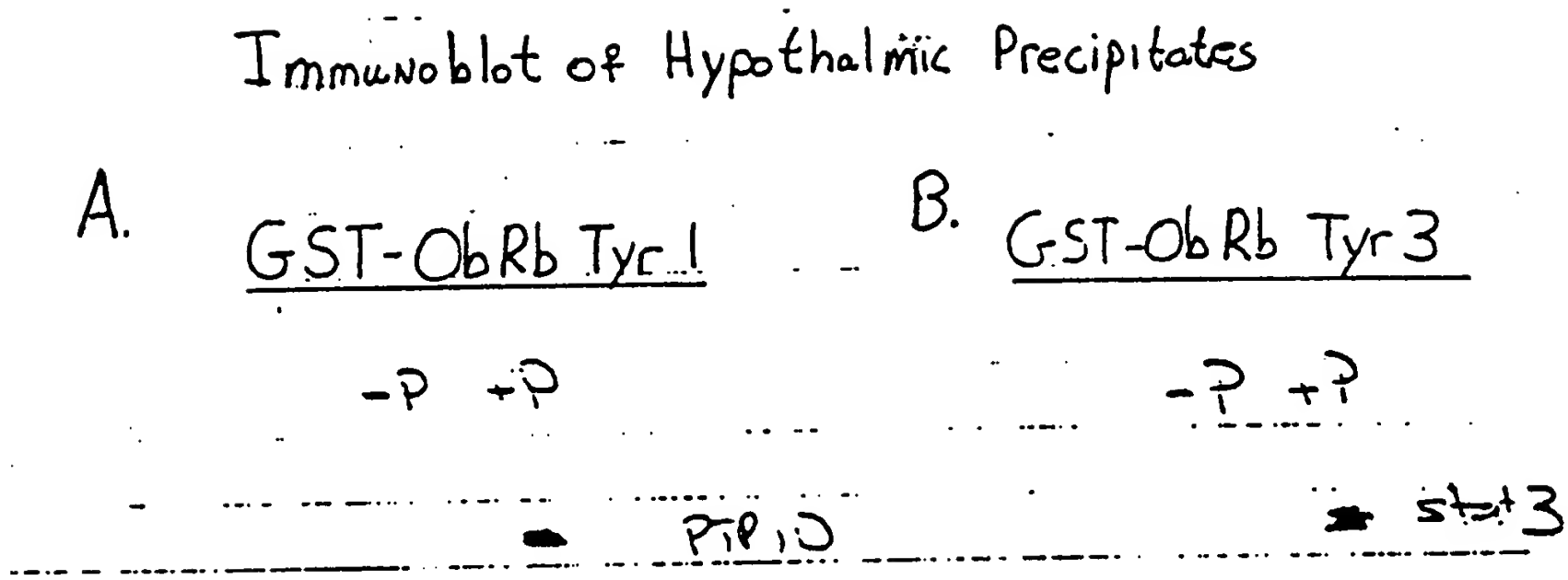
116
97
66
45

Figure 1B


Immunoblot of Hypothalmic Precipitates
A.
GST-ObRb Tyr 1
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B.
GST-ObRb Tyr 3
-P +P
Std

Figure 2

Involvement of Jak2 in Ob-Rb dependent PTP1D Activation

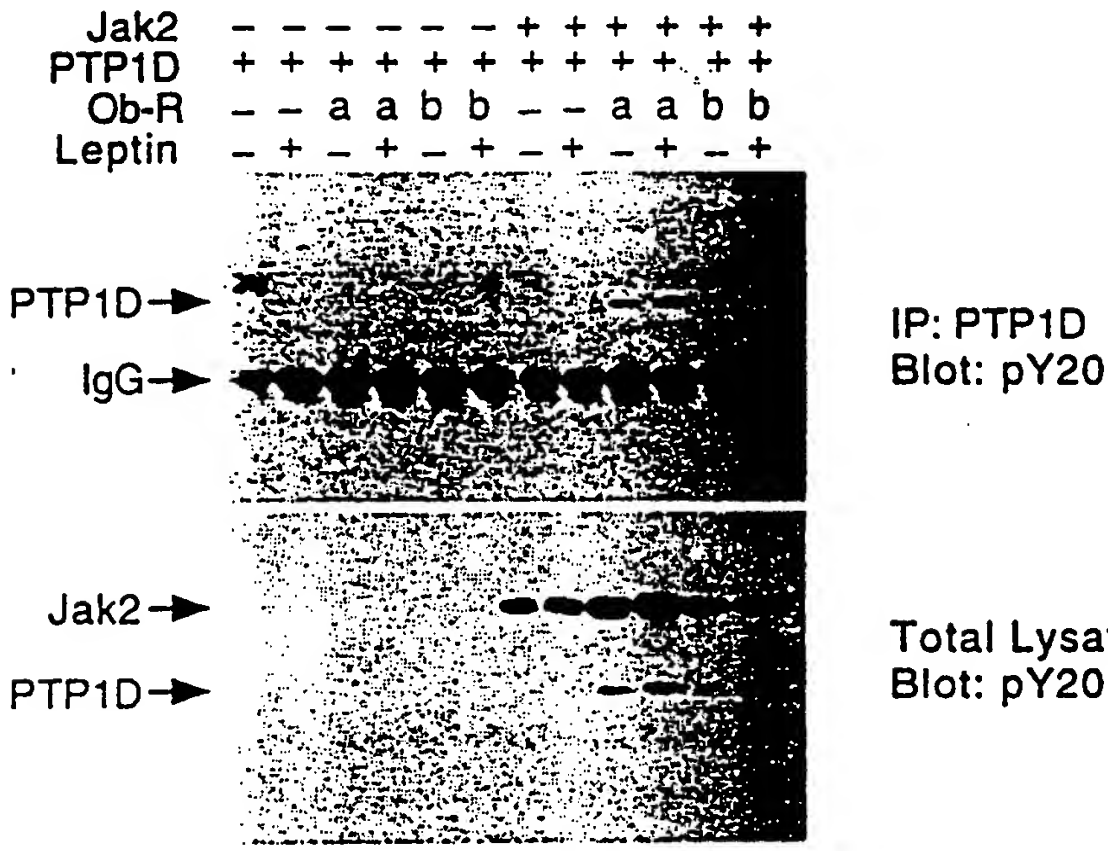

Figure 3

Time Course of Ob-Rb dependent PTP1D Activation

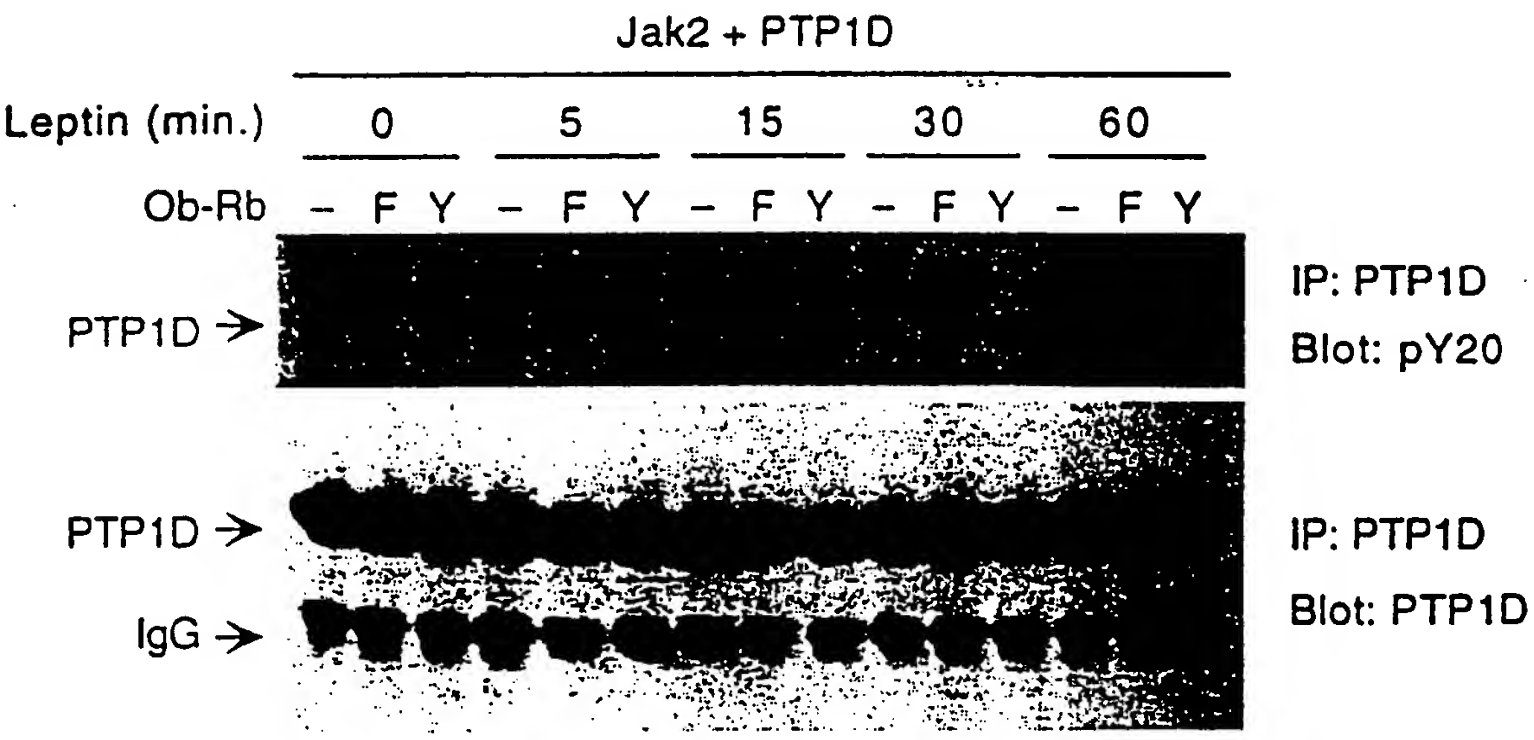

Figure 4

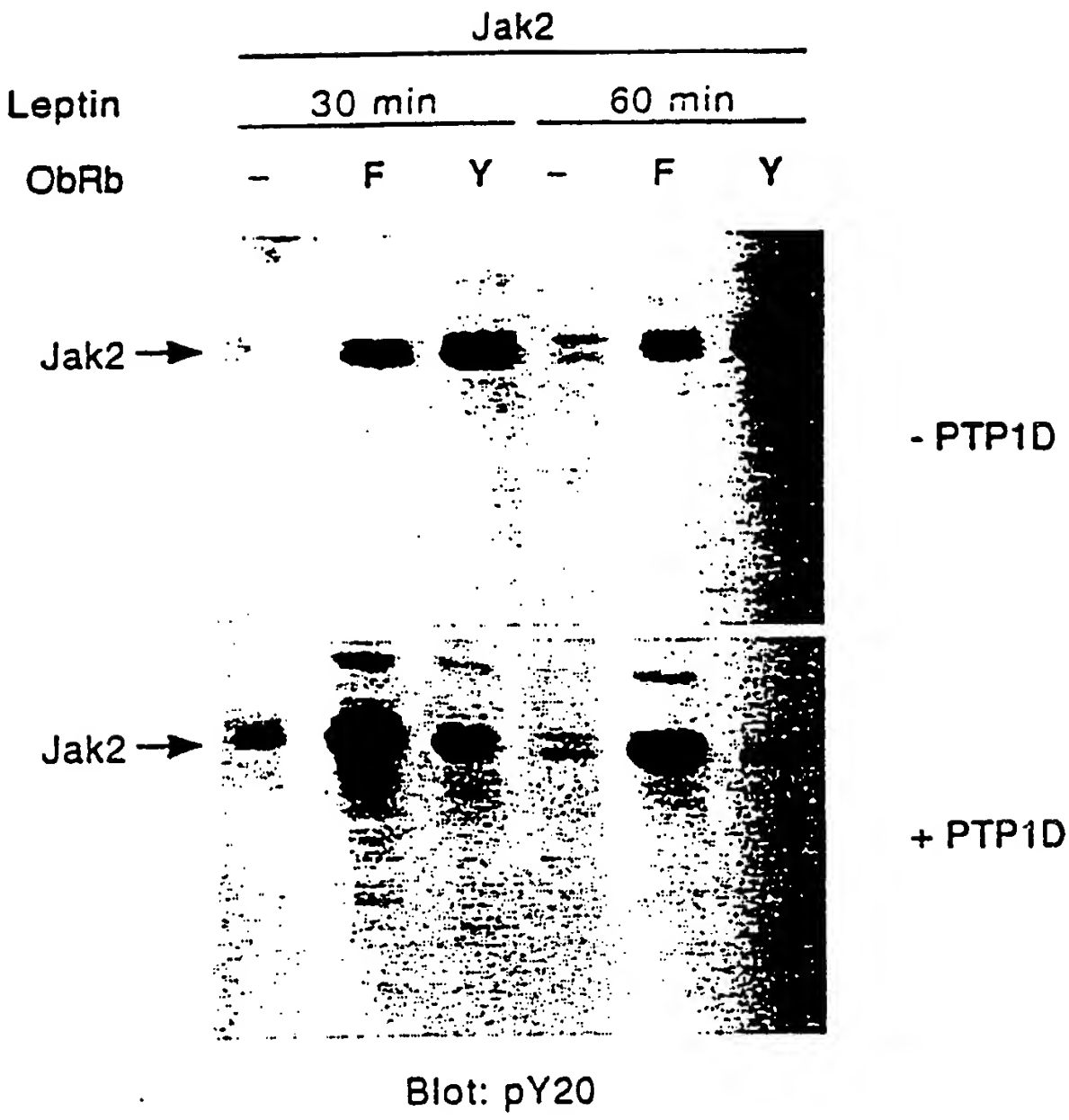
Jak2
Leptin
30 min
60 min
ObRb
–
F
Y
–
F
Y
Jak2
- PTP1D
Jak2
+ PTP1D
Blot: pY20

Figure 5

INTERNATIONAL SEARCH REPORT

Internat .al Application No
PCT/US 98/22797

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/68 G01N33/573 G01N33/74

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 26523 A (PROGENITOR INCORPORATED) 24 July 1997 see page 2, line 8 - line 9; claim 11 ---	1-20
A	WO 96 22308 A (ZYMOGENETICS INC & UNIVERSITY OF WASHINGTON) 25 July 1996 cited in the application see claim 1 ---	16,20
A	WO 96 38586 A (SMITHKLINE BEECHAM PLC) 5 December 1996 see the whole document ---	1-20
A	WO 94 08017 A (MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E.V.) 14 April 1994 see the whole document -----	1-20

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

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"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the International search	Date of mailing of the international search report
3 March 1999	10/03/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Van Bohemen, C

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat l Application No
PCT/US 98/22797

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9726523 A	24-07-1997	AU 1833197 A	11-08-1997
WO 9622308 A	25-07-1996	US 5827734 A	27-10-1998
		AU 4863196 A	07-08-1998
		US 5858967 A	12-01-1999
WO 9638586 A	05-12-1996	AU 6002396 A	18-12-1996
		CA 2222409 A	05-12-1996
		CN 1192248 A	02-09-1998
		CZ 9703790 A	13-05-1998
		EP 0832284 A	01-04-1998
		HU 9801744 A	28-10-1998
		NO 975504 A	28-11-1997
		PL 323638 A	14-04-1998
WO 9408017 A	14-04-1994	US 5589375 A	31-12-1996
		AU 5175793 A	26-04-1994
		CA 2146544 A	14-04-1994
		CN 1091469 A	31-08-1994
		EP 0663954 A	26-07-1995
		JP 8502885 T	02-04-1996
		MX 9306220 A	31-05-1996